

# Analytical Profiles of Drug Substances

Volume 9

*Edited by*

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## PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia, such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish *Analytical Profiles of Drug Substances* in a series of volumes of which this is the ninth.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physicochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not too distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. It is gratifying to note that increasingly profiles are being written not only in industrial laboratories but also academic institutions worldwide.

All those who have found the profiles useful are requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

The goal to cover all drug substances with comprehensive monographs is still a distant one. It is up to our perseverance to make it a reality.

Klaus Florey

# BACITRACIN

*Glenn A. Brewer*

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## 1. Introduction

The organism which produces bacitracin was isolated by Miss B.A. Johnson in June 1943 from the debrided tissue removed from a compound fracture of the tibia of a seven year old girl named Margaret Tracey<sup>1</sup>. Miss Johnson was working on a project directed by Dr. Frank L. Meleney. These workers thought that it might be possible to isolate an antibiotic producing organism from the mixed bacterial flora present in a severe wound.

A crude concentrate was soon produced, and in October, 1943 the first human clinical trial was started<sup>2</sup>. The process for the manufacture of bacitracin was scaled up and the first large scale clinical studies were reported in 1947<sup>3</sup>. Bacitracin was approved as a certifiable antibiotic in July 1949.

In 1944, Magarão and co-workers isolated a strain of Bacillus subtilis which had in vitro activity toward Mycobacterium tuberculosis<sup>4</sup>. The culture was studied in England and it was found that the culture no longer showed activity against Mycobacterium tuberculosis. Subsequently, a strain of Bacillus licheniformis was isolated from the culture and this isolate was found to produce an antibiotic which was called Ayfivin<sup>5</sup>. When the composition of bacitracin was better understood, it was realized that it and Ayfivin were probably identical, and the latter name was no longer used<sup>6</sup>.

Although bacitracin was known to be active primarily against Gram positive organisms, it was widely used in all types of infections. It was administered topically, by intramuscular injection, as lozenge for infections of the mouth and throat, intervaginally and as an ophthalmic preparation.

Apparently, as more potent preparations of bacitracin were produced, the material also increased in nephrotoxicity<sup>7</sup>. In a review on bacitracin published in 1952, the author states<sup>8</sup>:

"The side effects resulting from the administration of any therapeutic agent are of secondary importance in assessing the clinical value of the drug. They assume importance only if they limit either the dosage or duration of treatment because

of harmful effects on any organ or tissue of the body or any body function." This statement is interesting in the present era in which the importance of side effects practically eclipses the therapeutic activity and a potent therapeutic agent may be discarded because of relatively minor side effects.

Today, the U.S.P. recognizes bacitracin ointments for topical and ophthalmic use and sterile bacitracin for intramuscular injection<sup>9</sup>. In addition, the C.F.R. provides for the certification of bacitracin oral dosage forms and bacitracin combination products with other antibiotics and steroids for ophthalmic and topical use<sup>10</sup>.

It is probable that the veterinary use of bacitracin is more economically important than the clinical use, although volume figures are not readily available. The C.F.R. provides certification for bacitracin powder, the manganese and zinc salts and unrefined feed grade zinc bacitracin powder. In addition, bacitracin methylene disalicylate oral dosage forms, combination oral products with streptomycin sulfate, implantation pellets and a large variety of ophthalmic and topical dosage forms are monographed.

It is interesting to note that the number of publications on bacitracin chemistry and production have not waned in the thirty four years since the discovery of the antibiotic. It is rare to find a year in which a patent was not issued on the production of bacitracin, and the literature on the chemistry of the antibiotic continues to grow.

## 2. Chemistry

### 2.1 Structure

The key to the establishment of the structure of a natural product is the isolation of the pure substance. Counter-current distribution analysis was used by Craig and co-workers to demonstrate that at least three components were present in commercial bacitracin<sup>12</sup>. The major component was hydrolyzed and the following dipeptides were found:

phenylalanine and leucine

phenylalanine and ornithine.

In addition, phenylalanine, leucine, isoleucine,

glutamic acid, aspartic acid, lysine, histidine, cystine and ammonia were found by amino acid analysis using starch column chromatography. It was recognized by Craig and co-workers that some of the amino acids probably had the D-configuration, as racemic mixtures were isolated in some cases.

Newton and Abraham also used countercurrent distribution to study the purity of the antibiotic ayffivin<sup>6</sup>. They demonstrated that there were at least seven components in the mixture with the three major components being present in the ratio 4:1:4. Two components were shown to be identical to components in bacitracin and the name ayffivin was dropped (see section 1).

The same workers showed that at least ten components were present in crude bacitracin<sup>13</sup>. They were designated bacitracins E, D, B, A<sup>1</sup>, A, C, G, F<sub>1</sub> and F<sub>2</sub>. Bacitracins E, D, B and A showed a broad absorption band in the U.V. at 253 nm. Components C and G showed a sharper band at 250 nm while the three F components had a broad maximum at 288 nm. They established that all the components contained cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, phenylalanine and leucine (or isoleucine). Bacitracin C also contained a component which was not separable from glycine in the chromatographic system used, while the bacitracins B, D and E yielded valine. Bacitracins D and E apparently do not contain amide groupings while A, B, C, G and the F components do.

Newton and Abraham continued their examination of the structure of bacitracin A, the major component of the complex<sup>14</sup>. They established that the antibiotic had three basic centers, one amide and had a unit molecular weight of 1500.

In addition, they established that each unit contained two carboxyl, one  $\alpha$ -amino, one  $\delta$ -amino and one histidine glyoxaline as ionizable groups. Bacitracin A did not contain a disulfide linkage, but a thiol group was liberated on acid hydrolysis. An amide group was also liberated and the ultraviolet absorbance at 254 nm disappeared on acid hydrolysis. On hydrogenation with Raney nickel, the group which contained the cystine residue was converted to an alanine residue. The authors postulated that

bacitracin A contains a thiazoline ring.

Craig and co-workers used their newly developed ion exchange amino acid analyzer to establish the amino acid composition of bacitracin A<sup>15</sup>. The same group established a molecular weight of 1470 for bacitracin A using a partial substitution method<sup>16</sup>. They also proposed a cyclic structure for the molecule.

Ingram reported that bacitracin A contained no free amino end group based on methylation studies<sup>17</sup>.

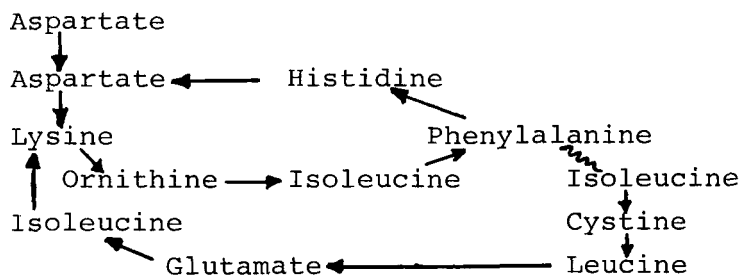
Porath, using partial acid hydrolysis, established the amino acid sequence for the ring as glutamic acid, cysteine, isoleucine, ornithine, histidine and 2 moles of aspartic acid<sup>18</sup>. He postulated that the sulfur of cysteine was involved in a hetero cyclic ring between lysine and glutamic acid. An unidentified ninhydrin-negative compound is attached to lysine.

Lockhart, Newton and Abraham performed acid hydrolysis at 37°C<sup>19</sup>. They found the amino acid sequences:

isoleucine-cysteine-leucine-glutamic acid and  
ornithine-phenylalanine-isoleucine.

The latter peptide appeared to be an N-terminal peptide.

Lockhart and Abraham postulated the following partial structure for bacitracin A<sup>20</sup>.



They also indicated that the sequence lysine-ornithine-valine-phenylalanine occurs in bacitracin B.

Craig and co-workers confirmed the presence of three isoleucine residues in bacitracin A and, on this basis, postulated the empirical formula  $C_{66}H_{168}O_{14}N_{17}S$  for the antibiotic<sup>21</sup>.

The same workers proposed the following structure for bacitracin A based on the products obtained after partial hydrolysis<sup>22,23,24</sup>.

Isoleucine-cysteine-leucine-glutamic acid-isoleucine-lysine

Aspartic acid-aspartic acid-histidine-phenylalanine-isoleucine-ornithine

It should be noted that this structure differs significantly from the one proposed by Abraham's group<sup>20</sup>, and does not explain their earlier findings<sup>14</sup>.

Craig and co-workers proposed that bacitracin A contains a thiazole ring formed by the condensation of cysteine and isoleucine<sup>25</sup>. They began a study of the chemistry of bacitracin F.

Further studies by Abraham and co-workers confirmed the fact that there were three isoleucine residues in bacitracin A<sup>26,27</sup>. This had been previously indicated by Craig and co-workers<sup>21</sup>.

Lockhart and Abraham concluded that the lysine residue in bacitracin A is linked to isoleucine through the  $\alpha$ -amino group and to aspartic acid through the  $\epsilon$ -amino group<sup>28</sup>. This aspartic acid residue has the L-configuration while the other aspartic acid in bacitracin A has the D-configuration.

Wrinch proposed a structure for bacitracin A based on the published information<sup>29</sup>.

Several reviews of the chemistry of bacitracin A have been published<sup>30,31,32,33,34</sup>.

Craig and Konigsberg established that bacitracin F was a degradation product of bacitracin A<sup>35</sup>. The conversion was accompanied by the loss of



ammonia.

Swallow and Abraham found that the glutamic acid residue was connected via the  $\alpha$ -carboxyl group and that the  $\gamma$ -carboxyl group is free<sup>36</sup>. One of the aspartic acid residues was present as an amide.

Stoffel and Craig synthesized a number of cysteine peptides modeled on the N-terminal portion of bacitracin A<sup>37</sup>. They hoped to establish the substitution that would give stable thiazoline rings.

Craig and co-workers studied the acid isomerization of bacitracin A<sup>38</sup>. The transformation involves the epimerization of the N-terminal isoleucine residue.

Theodoropoulos established that both lysine residues in bacitracin A are  $\alpha$ -isoleucyl-( $\epsilon$ -aspartyl)-lysine<sup>39</sup>.

Kaneko and co-workers published a series of papers on the synthesis of peptide intermediates to be used in the total synthesis of bacitracin A<sup>40,41,42,43,44,45,46</sup>.

Ratti and co-workers established the optical configuration of the aspartyl and asparaginyl residues of bacitracin A as D and L respectively<sup>47</sup>.

Cornell and Guiney established that the coordination sites for zinc in bacitracin were the thiazolene ring and histidine residue<sup>48</sup>.

Manning developed a method to establish the amount of racemization that occurred during acid hydrolysis<sup>49,50</sup>.

On the basis of NMR studies, a space-filling model of bacitracin A was proposed<sup>51</sup>.

The presently accepted structures for the bacitracins can be found in Section 3.

## 2.2 Biosynthesis

The cell-free enzymatic synthesis of bacitracin A has been extensively studied by a number of workers.

Bernlohr and Sievert noted the similarity of the amino acid composition of bacitracin and Bacillus licheniformis spore coats<sup>52</sup>. This suggested that the antibiotic was a precursor of a structural entity of the bacterial cell.

Bernlohr and Novelli indicated that bacitracin was produced by postlogarithmic cells of Bacillus licheniformis which are in the process of producing spores<sup>53</sup>. The amino acids were not incorporated into bacitracin by a normal mechanism.

Shimura and co-workers found that the amount of bacitracin produced by B. licheniformis was governed by the amount of cysteine present in the medium<sup>54</sup>.

Cornell published a thesis on the biosynthesis of bacitracin<sup>55</sup>.

The cell-free synthesis of bacitracin was first achieved by Shimura and co-workers<sup>56</sup>. They utilized lysed protoplasts of B. licheniformis. The incorporation of L-histidine was inhibited when various D-amino acids were added. The biosynthesis was not inhibited by ribonuclease, chloramphenicol or puromycin so it was concluded that the biosynthetic pathway was different from that involved in protein biosynthesis.

Pfaender also reported the biosynthesis of bacitracin with a cell-free preparation<sup>57</sup>. He found that leaving out one of the required amino acids or the substitution of a D-amino acid for an L-amino acid stopped the synthesis.

Pfaender and co-workers fractionated the enzyme system and found two fractions with molecular weights of 200,000 and 350,000, which dissociated to 50,000 units on storage for one day in the cold<sup>58</sup>.

Froyshov and Laland purified bacitracin synthetase about 11-fold<sup>59</sup>. They showed that two fractions were present, both of which were required for the synthesis of bacitracin. The amino acids required for the pyrophosphate-ATP exchange reactions were determined for each fraction.

Froyshov reported that he had resolved

bacitracin synthetase into three fractions by affinity chromatography<sup>60</sup>.

Ishihara and Shimura purified bacitracin synthetase 25-fold<sup>61</sup>.

Froyshov continued his work and found that fraction A was responsible for the chain lengthening of bacitracin A<sup>62</sup>.

Ishihara published a review on the biosynthesis of bacitracin A with cell-free enzyme preparations<sup>63</sup>.

Froyshov also reviewed progress in cell-free biosynthesis of bacitracin A<sup>64</sup>.

Wang and co-workers<sup>65</sup> and Umezawa and co-workers<sup>66</sup> have published reports on the practical cell-free synthesis of bacitracin A.

### 3. Description

#### 3.1 Composition, Formula, Molecular Weight

The bacitracin of commerce is a mixture of components. The major component is bacitracin A.

The mixture of bacitracin components [1405-87-4] will be referred to in this monograph as bacitracin. Certain salts and derivatives of the bacitracin complex have been utilized in feeds or formulations

Zinc bacitracin [1405-89-6]

Manganese bacitracin [1405-99-8]

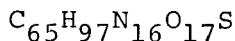
Sodium bacitracin [39436-06-1]

Methylenebis [2-hydroxybenzoate]-[55852-84-1]

#### 3.11 Bacitracin A [22601-59-8]

The structure of bacitracin A was elucidated after almost twenty years of work by a number of different groups (Section 2). It is no wonder then that there is disagreement in the literature on which group established the definitive structure. Ressler and Kashelkar, using a dehydration-reduction technique established the final position of the amino acids in the seven membered





Molecular Weight 1406.66

### 3.14 Other Bacitracin Components

A number of other minor components have been identified in the bacitracin complex. The structures of these components are not known at the present time.

Bacitracin B <sub>1</sub>	(57762-79-5)
Bacitracin B <sub>2</sub>	(57762-78-4)
Bacitracin C <sup>2</sup>	(1403-00-5)
Bacitracin D	(1403-01-6)
Bacitracin E	(1403-07-7)
Bacitracin F <sub>1</sub>	(1403-04-9)
Bacitracin F <sub>2</sub>	(1403-05-0)
Bacitracin G	(1403-03-8)

Unless otherwise specified, in the remainder of this profile when we use the name bacitracin we refer to the bacitracin complex.

## 4. Physical Properties

### 4.1 Spectra

#### 4.11 Infrared Spectrum

The infrared spectrum of bacitracin has been published by Hayden and co-workers<sup>70</sup>.

The infrared curves of bacitracin and zinc bacitracin taken as mineral oil mulls and as KBr pellets are shown in Figures 1-4<sup>71</sup>.

#### 4.12 Nuclear Magnetic Resonance Spectrum

Chapman and Golden used NMR to study deuterium exchange in bacitracin A<sup>51</sup>. This work along with the tritium exchange studies conducted by Craig and co-workers<sup>68</sup> established the conformation of bacitracin A in aqueous solution.

Coates and co-workers used 270 MHz NMR to measure proton spin lattice relaxation times for bacitracin A<sup>72</sup>.

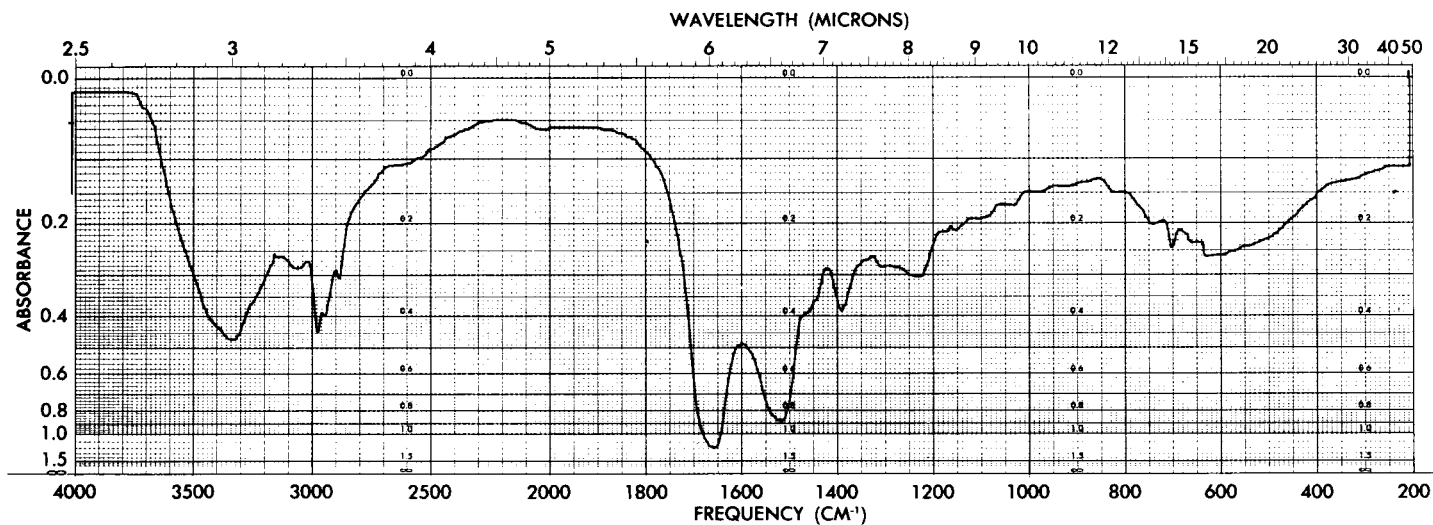


Figure 1. Infrared Spectrum of Bacitracin-KBr Pellet

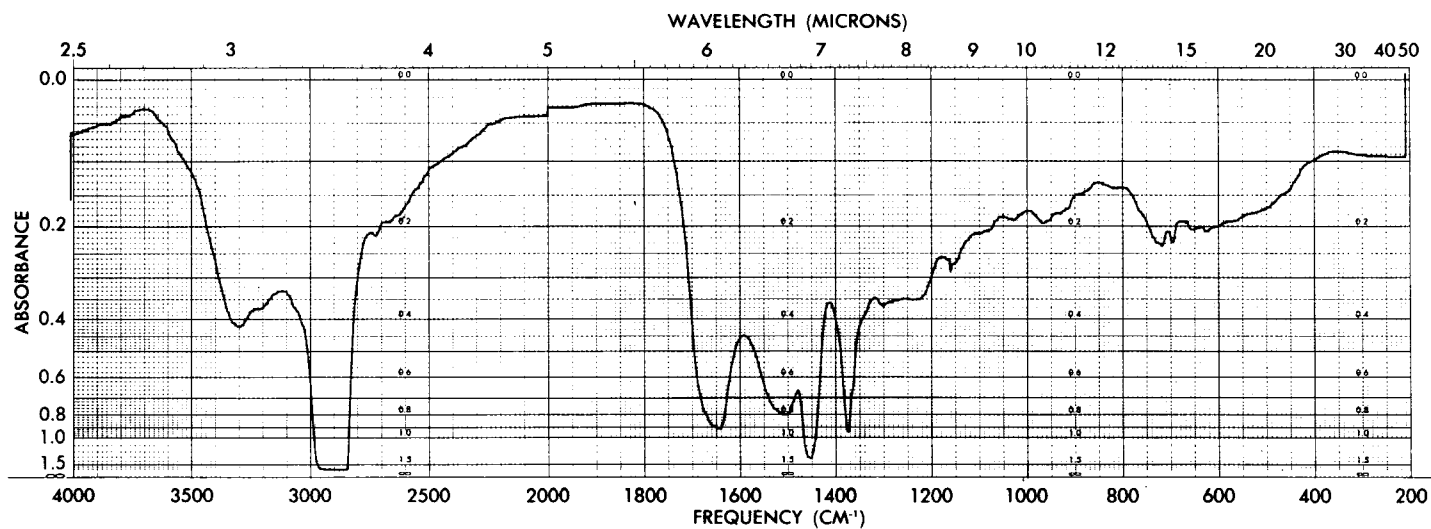


Figure 2. Infrared Spectrum of Bacitracin-Mineral Oil Mull

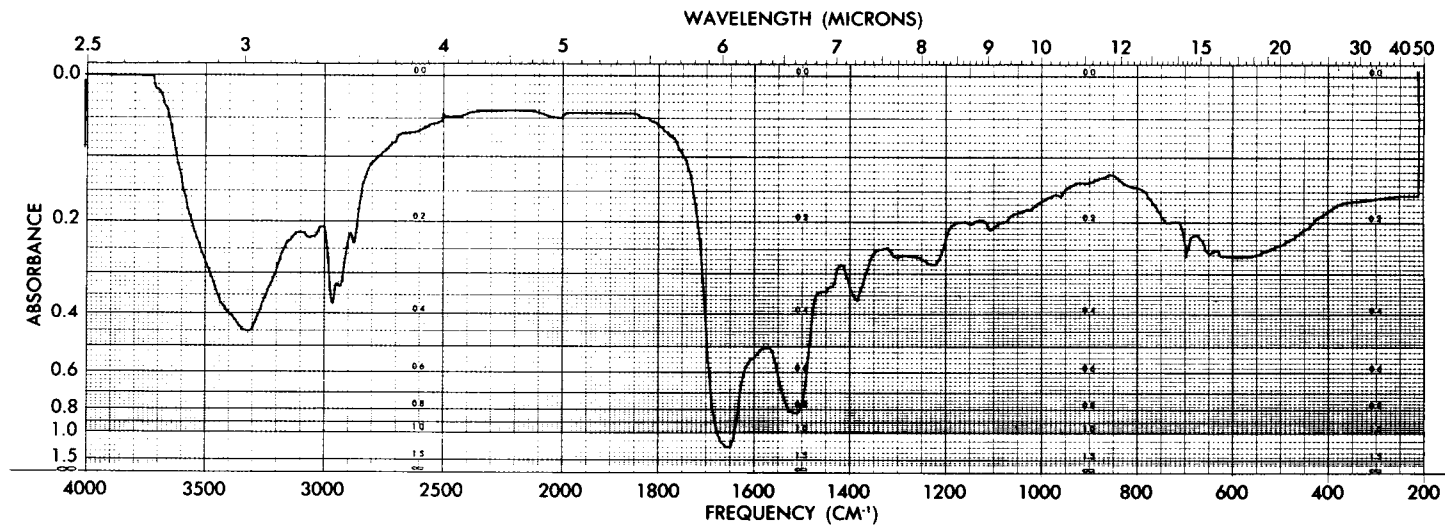


Figure 3. Infrared Spectrum of Zinc Bacitracin-KBr Pellet



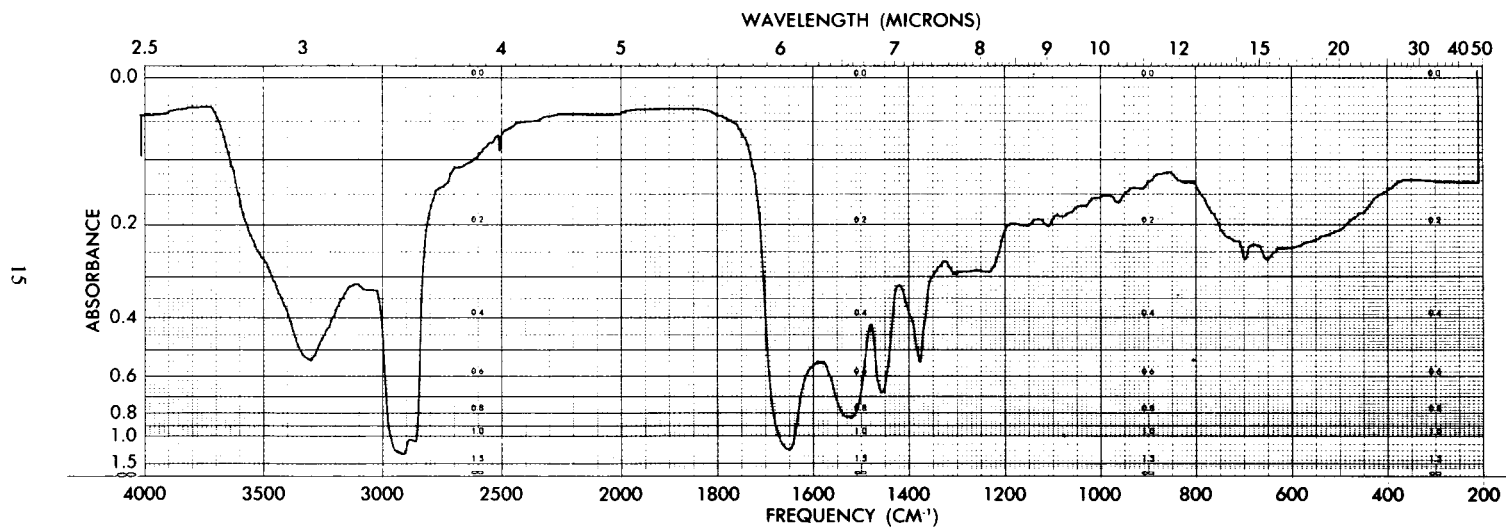


Figure 4. Infrared Spectrum of Zinc Bacitracin-Mineral Oil Mull

Reynolds and co-workers used  $\text{Cl}^{35}$  magnetic resonance spectroscopy to establish the tautomeric equilibrium of the histidine ring in bacitracin A<sup>73</sup>.

The NMR spectrum of bacitracin in  $\text{D}_2\text{O}$  is shown in Figure 5<sup>74</sup>.

#### 4.13 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of bacitracin was reported by Hayden, *et al.*<sup>70</sup>.

The ultraviolet spectrum of bacitracin was determined in water, methanol, dilute acid and dilute alkali<sup>75</sup>. In all solvents, a small peak with an  $E(1\%, 1 \text{ cm})$  of about 20 was exhibited at about 250 nm. There was no significant shift in wavelength or decrease in absorbance on standing in dilute acid or alkali for periods up to 24 hours at room temperature.

#### 4.14 Fluorescence Spectrum

Bacitracin exhibits a very weak fluorescence in aqueous solution<sup>76</sup>. In both acid and alkaline solutions the excitation wavelength is at about 292 nm and the emission occurs at about 325 nm.

#### 4.15 Acoustic Absorption Spectrum

Slutsky, Madsen and White determined the acoustic absorption spectrum of bacitracin and other peptides<sup>77</sup>.

### 4.2 Crystal Properties

#### 4.21 X-Ray Powder Diffraction

Samples of U.S.P. Reference standard of bacitracin and zinc bacitracin were examined by powder x-ray diffraction. Both substances were found to be amorphous as indicated by the absence of any peaks in the x-ray pattern<sup>78</sup>.

#### 4.22 Hygroscopicity

Hayashi and co-workers determined

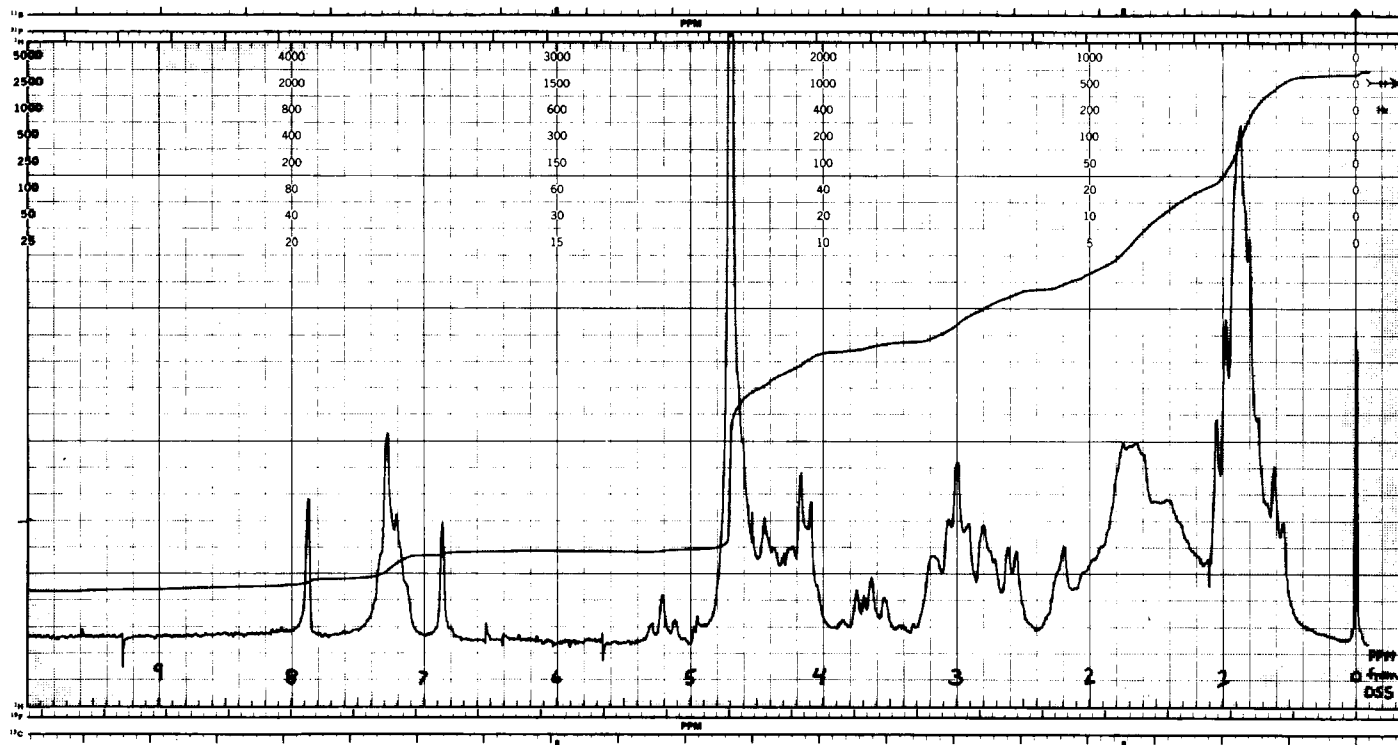


Figure 5. NMR Spectrum of Bacitracin in D<sub>2</sub>O

the hygroscopicity of bacitracin at 100%, 93% and 63% relative humidity<sup>82</sup>.

Lanning also reported on the hygroscopicity of bacitracin<sup>83</sup>.

### 4.3 Solubility

#### 4.31 Solubility in Pure Solvents

Weiss, Andrew and Wright published data on the solubility of bacitracin and zinc bacitracin in a number of solvents<sup>79</sup>.

<u>Solvent</u>	<u>Solubility in mg/ml</u>	
	<u>Bacitracin</u>	<u>Zinc Bacitracin</u>
water	>20	5.1
acetone	0.75	1.0
1,4-dioxane	0.70	0.49
ethanol	9.1	2.0
ethylene glycol	>20	7.95
formamide	19.9	>20
isopropyl alcohol	1.85	0.16
methanol	>20	6.55
pyridine	9.15	4.05
benzene	0.025	0.065
benzyl alcohol	>20	10.35
carbon disulfide	0.30	0.30
carbon tetrachloride	0.18	0.12
chloroform	0.0	0.01
cyclohexane	0.075	0.06
ethyl acetate	0.047	1.3
diethyl ether	0.065	0.02
ethylene chloride	0.025	1.1
isoamyl alcohol	1.65	2.6
isooctane	0.55	0.015
methyl ethyl ketone	0.20	0.85
petroleum ether	0.35	0.025
toluene	0.15	0.02
isoamyl acetate	0.09	0.45

Gross noted that bacitracin is more soluble in aqueous solution in the pH range 6.5 to 7.5<sup>80</sup>.

#### 4.32 Distribution Coefficient

Carpenter and co-workers determined the distribution coefficient of bacitracin in 2-butanol-0.1N acid<sup>81</sup>.

#### 4.33 Formulation Release

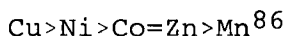
Nesbit and co-workers determined the release of bacitracin from ointment bases<sup>84</sup>.

### 4.4 Physical Properties of Solutions

#### 4.41 Metal Binding

Selzer noted that while most antibiotics contain less than 30 p.p.m. of heavy metals, bacitracin, by virtue of salt formation, may contain more than three times this concentration<sup>85</sup>.

Garbutt, Morehouse and Hansen established the following order for complex formation of metal salts and bacitracin:



All the metals, except manganese, complexed the bacitracin group which titrates between 5.5 and 7.5. Using titration data and the U.V. spectra of the complexes, these workers postulated the involvement of the imidazole group of histidine in the complex.

Using NMR and ORD measurements, Cornell and co-workers found that zinc complexes between the thiazoline and histidine residues<sup>48</sup>.

Weinberg measured the stability constants of the binding of copper, nickel, cobalt, zinc and manganese to bacitracin<sup>87</sup>.

Storm and Strominger established the association constants for bacitracins A and F with magnesium<sup>88</sup>. Bacitracin F has a lower association constant.

#### 4.42 Optical Rotary Dispersion

Konigsberg and Craig reported that bacitracin undergoes a change in rotation

below pH 4 due to the epimerization of the terminal isoleucine group<sup>89</sup>.

Craig reported on O.R.D. studies of bacitracin A<sup>90</sup>.

Cornell and co-workers used O.R.D. to study the attachment of zinc to bacitracin A<sup>48</sup>.

Craig and co-workers studied the conformation of bacitracin A in aqueous solution<sup>91</sup>.

#### 4.43 Isoelectric Point

Messing patented a method to determine the isoelectric point of proteins<sup>92</sup>. The method was used to establish the isoelectric point of bacitracin as 8.8. This value agrees well with a determination of 8.5 using electrophoresis.

#### 4.44 Dialysis

Craig and co-workers developed the technique of thin-film dialysis to study the conformation of large molecules in solution<sup>90</sup>. Bacitracin A was one of the model compounds studied.

Klein and co-workers used bacitracin as a model compound to establish the properties of four cellulosic membranes<sup>93</sup>.

Craig and co-workers reported additional studies on the dialysis of bacitracin A<sup>94</sup>.

Krogerus used dialysis to study the release rate of bacitracin from various ointment bases<sup>95</sup>.

Several reviews have been published on the physical and chemical properties of the bacitracins<sup>96,97,98</sup>.

### 5. Production

#### 5.1 Microbiological

Meleney and co-workers described the production of bacitracin on L-glutamic acid

synthetic and soybean digest media<sup>99</sup>.

Hendlin studied the formation of bacitracin by Bacillus subtilis and evaluated the effect of the addition of various ions, organic acids, amino acids and carbohydrates<sup>100</sup>.

Inskeep and co-workers described a new plant built for the production of bacitracin<sup>101</sup>.

Darker patented the addition of various salts to soybean medium to stimulate production of bacitracin<sup>102</sup>.

Keko, Bennett and Arzberger patented a soybean meal-starch medium for the production of bacitracin<sup>103</sup>.

Su and Lu noted the increased production of bacitracin in a peanut oil meal-starch medium when calcium lactate and potassium phosphate were added<sup>104</sup>.

Cohen patented a soybean meal-dextrin medium for the production of bacitracin<sup>105</sup>.

Wilk specified the pH ranges for the growth and antibiotic production phases of a bacitracin-producing culture<sup>106</sup>.

Freaney and Allen patented a fermentation medium capable of supporting a yield of about 320 units/ml in 24 hours<sup>107</sup>.

Ziffer patented a soybean-sucrose medium for bacitracin production<sup>108</sup>.

Ripoli published a report on the production of bacitracin in five-liter flasks<sup>109</sup>.

Siquiroff found that the production of bacitracin was higher in surface culture than in shaken flasks<sup>110</sup>.

Zorn patented a fermentation medium containing a water-soluble salt of cobalt<sup>111</sup>. He proposed that cobalt complexes of bacitracin were formed which stabilized the bacitracin for use in animal feed supplements.

Aida and Ito describe the formation of bacitracin A and bacitracin X complex from bacterial protoplasts (see Section 2.2)<sup>112,113,114,115,116</sup>. Bacitracin X complex has a similar amino acid composition to bacitracins A and B but can be separated by paper chromatography.

Cornell and Snoke showed by adding various antibiotics and D-phenylalanine that the biosynthesis of protein and bacitracin by Bacillus licheniformis was accomplished by different metabolic pathways<sup>117</sup>. The same workers showed that B. licheniformis is inhibited by bacitracin in the early stages of growth<sup>118</sup>.

Brandl and co-workers studied oxygen transfer in the bacitracin fermentation<sup>119</sup>.

Weinberg and Tonniss showed that although inhibitors of nucleic acid metabolism, messenger RNA synthesis and protein synthesis inhibited the production of bacitracin, the inhibition could be overcome by the addition of a manganese salt<sup>120</sup>.

Weinberg postulated the function of the bacitracin peptide and other peptide antibiotics for Bacillus species<sup>121</sup>.

Styczynska and co-workers noted that the production of bacitracin by Bacillus subtilis was stimulated when fermentation was conducted as a mixed culture process with a Pseudomonas strain<sup>122</sup>.

Lubinski patented a process using a strain of Bacillus subtilis adapted to iron and grown on a soy-fish meal medium<sup>238</sup>.

Feuer and co-workers obtained a patent on an antifoam composition which was useful in the bacitracin fermentation<sup>123</sup>.

Chigaleichik and co-workers defined a synthetic medium for bacitracin production by Bacillus polymyxa<sup>124</sup>.

Simlot, Pfaender and Specht noted that changes in the fermentation medium did not alter the quantity of bacitracin synthesized but did change the type produced<sup>125</sup>.



Haavik suggested that glucose inhibited the formation of bacitracin primarily by lowering the pH of the fermentation, and not by catabolite repression control<sup>126,127</sup>. The same worker found that phosphate only has an adverse effect when it alters the optimum pH of the fermentation<sup>128</sup>.

Haavik postulated that bacitracin may participate in manganese-ion transport through the cell membrane of Bacillus licheniformis<sup>129,130,131,132</sup>.

Kurima and co-workers patented a process for the production of bacitracin<sup>133</sup>.

Pass and Raczynska-Bojanowska found that high bacitracin-producing strains of Bacillus subtilis lack ornithine  $\delta$ -transaminase<sup>134,135</sup>. If ornithine is added to low producing strains, their productivity is increased.

Vitkovic and Sadoff found that bacitracin is a constituent of vegetative cell protein<sup>136</sup>.

Makukhina and co-workers described the production of bacitracin<sup>137</sup>.

Tyc and co-workers have patented a process for the production of bacitracin utilizing a non-sporulating strain<sup>138</sup>.

Lipavska and associates used acriflavine to prevent infection of Bacillus licheniformis with bacteriophage BLE<sup>139</sup>. They found that acriflavine did not inhibit the production of bacitracin.

Tyc and Kadzikiewicz described their method of producing ultraviolet mutants of Bacillus licheniformis, and evaluating selected isolates for bacitracin production<sup>140</sup>. Increases of 50 to 75% were obtained with four isolates.

Haavik studied the metabolism of a high yielding mutant strain of B. licheniformis and found that the addition of L-leucine stimulated bacitracin production<sup>141</sup>.

Raczynska-Bojanowska and co-workers patented a process for the simultaneous production of

bacitracin and proteases<sup>142</sup>.

## 5.2 Isolation

Anker and co-workers used butanol extraction to isolate the bacitracin from the fermentation broth<sup>99</sup>.

Gorley used ammonium sulfate salt fractionation to purify crude bacitracin<sup>143</sup>.

Johnson and Meleney patented a process for the production and recovery of bacitracin<sup>144</sup>.

There are four common ways in which bacitracin is isolated from fermentation broth. A number of patents and papers have been published on these.

### 5.21 Precipitation From Broth

Various workers have used salts to precipitate bacitracin from the fermentation broth. After the bacitracin salt mixture is filtered off, the pH is adjusted and the antibiotic is extracted into a solvent<sup>145,146,147,148,149,150,151,152,153,154,155,156,157,158,159,160</sup>.

### 5.22 Ion Exchange of Bacitracin

A number of patents have been issued for processes which involve the removal of bacitracin from broth by means of an ion exchange column<sup>161,162,163,164,165,166,167,168,169,170,171,172</sup>.

### 5.23 Solvent Extraction of Bacitracin

Solvent extraction has been used less extensively than the first two methods based on patents issued <sup>99,173,174,175</sup>. Apparently, the development of this isolation procedure has been carried out primarily by one company.

### 5.24 Metal Salts of Bacitracin

The metal salts of bacitracin are used extensively as animal feed supplements (See Section 1). These insoluble salts can be formed directly in the fermentation broth and isolated as a

crude concentrate for animal feed use<sup>177,178,179,180,181,182,183</sup>.

## 5.25 Miscellaneous Methods

Namiki has published a report on a method used to isolate high potency bacitracin<sup>184</sup>.

Monroe and Ward have patented a process to precipitate bacitracin on diatomaceous earth<sup>185</sup>. The dried solid can be used as an animal feed supplement.

Ores and Rauber have used the non-ionic resin XAD-2 to isolate bacitracin<sup>186</sup>.

Kindraka and Gallagher have used ultrafiltration to remove bacitracin from fermentation broth<sup>187</sup>.

Malitskii and Mikhel'son have noted that dry bacitracin has tendency to undergo spontaneous combustion<sup>188</sup>.

Brecka and co-workers inoculated a bacitracin fermentor with Rhodotorula flava after the antibiotic was produced<sup>189</sup>. The fermentor contained both bacitracin and  $\gamma$ -carotene at harvest. The use of the second fermentation was to remove fermentation by-products.

Stepanov and Rudenskaya have used immobilized bacitracin to purify proteolytic enzymes<sup>190</sup>.

## 6. Stability

### 6.1 Stability of Solid

Bond, Himelick and MacDonald reported that bacitracin was stable at temperatures up to 37°C<sup>191</sup>. Craig and co-workers also indicated that bacitracin is relatively stable as a solid<sup>192</sup>. Gross studied the stability of bacitracin powder at temperatures up to 60°C<sup>193</sup>. He indicated that after a minor initial drop, the preparations were relatively stable. There was no difference in stability between high and low potency preparations.

Babin , Coustou and Brisou showed that bacitracin in a mixture with papain enzyme powder maintained its potency for a six month period<sup>194</sup>.

Gupta, Vyas and Sekhon showed that 15 Mrads of neutron and  $\gamma$ -radiation did not change the activity of bacitracin powder<sup>195</sup>.

Tsuji and Robertson also showed that <sup>60</sup>Co radiation did not cause potency loss of bacitracin powder<sup>196</sup>. Ethylene oxide treatment caused 46% reduction in potency, but did not cause the formation of bacitracin F. Dry heat sterilization caused a 35% decrease in potency with a corresponding increase in bacitracin F.

## 6.2 Stability of Solutions

Anker and co-workers reported that solutions of bacitracin were stable for 8 to 12 months at 5°C<sup>99</sup>. Hayashi and co-workers found that a solution of bacitracin in pH 7 phosphate buffer lost 25% of the initial potency after 6 days at room temperature<sup>82</sup>.

Vasilescu and Molsă<sup>✓</sup> found that solutions of bacitracin were most stable at pH 4.4<sup>98</sup>.

Craig and Konigsberg showed that bacitracin B was inactivated more rapidly than bacitracin A<sup>35</sup>. In both cases, bacitracin F was a major decomposition product.

The same workers showed that below pH 4.0 bacitracin undergoes an epimerization of the terminal isoleucine residue<sup>89,38</sup>.

Pirila, Saukkonen and Santaoja separated the degradation products of bacitracin in solution<sup>197</sup>.

Herrmann, Woodward and Pulaski postulated that the inactivation of bacitracin on passage through the gastrointestinal tract of rats is due to degradation<sup>198</sup>.

Pirila, Salo and Pirila found that the complex of bacitracin with sodium dodecyl sulfate was stable in solution, although the complex showed

diminished skin penetration<sup>199</sup>.

Makinen found that bacitracin inhibits the activity of papain, subtilisin and leucine aminopeptidase<sup>200</sup>.

### 6.3 Light Stability

Wurtzen found that exposure to sunlight and temperature variations between 20°C and 35°C caused 20-35% loss of activity in 6 days<sup>201</sup>.

### 6.4 Formulation Stability

Bond and co-workers reported that anhydrous grease based ointments were stable while water miscible ointments were not<sup>191</sup>. A number of other investigators agree with these findings<sup>82,202</sup>.

Hegarty and Verwey patented formulations for bacitracin that were stable<sup>203</sup>.

Plaxco and Husa established the stability of bacitracin in a number of ointment bases<sup>204</sup>. Other authors evaluated various other formulation excipients<sup>205,206,207,208,209,239</sup>.

Gordon patented aerosol compositions of bacitracin<sup>210</sup>.

Snyder patented a stable formulation of bacitracin in animal feed<sup>211</sup>. The bacitracin was coated with oil and the droplets absorbed on diatomaceous earth to form a free-flowing powder.

Saito, Kawano and Ichijima patented a bacitracin feed additive stabilized with 2-oxo-4-methyl-6-ureidohexahydropyrimidine<sup>212</sup>.

### 6.5 Stability of Metal Salts

Gross, Johnson and Lafferty showed that zinc bacitracin was more stable than bacitracin in troches, ointments and tablets<sup>213</sup>. Other additives have confirmed the increased stability of salts of bacitracin with zinc and other metals<sup>214,215,216,217,91</sup>.

Crisler and Weinberg indicated that

while zinc salt of bacitracin was not more stable than bacitracin to autoclaving, the salt enhances the antibiotic activity of bacitracin 11-fold<sup>218, 219</sup>.

Tanaka, Seki and Ito patented the use of mineral salts of bacitracin as animal feed supplements<sup>220</sup>. These salts were reported to have enhanced stability. Other patents have been issued on the use of metal salts<sup>221, 222</sup>.

## 7. Analytical Methods

### 7.1 Identity Tests

#### 7.11 Physical Methods

Landgren differentiated antibiotics by measuring the refractive index of the crystals using liquids of known refractive index<sup>223</sup>.

Zief and co-workers prepared the tetraphenylboron derivatives of several antibiotics<sup>224</sup>. The melting points of these derivatives were used to identify them.

Matta and co-workers also utilized the tetraphenyl borate derivative for antibiotic identification<sup>225</sup>.

#### 7.12 Colorimetric Tests

Fischbach and Levine utilized the ninhydrin reaction as an identity test<sup>226</sup>.

Hayashi and co-workers reported that bacitracin gave positive biuret, Adamkewitz, Millon and Molisch reactions<sup>82</sup>. Wornick and Kuhn indicated that bacitracin produces a violet color with ninhydrin spray on paper<sup>227</sup>.

#### 7.13 Chromatographic Methods

Almost any chromatographic system for bacitracin could be used as an identity test for the antibiotic. In this section we are listing those systems specifically indicated as identity tests, other chromatographic methods can be found in Section 7.4.

<u>Type of Support</u>	<u>Solvent System</u>	<u>Detection</u>	<u>Reference</u>
Paper		Microbiol.	228
TLC	AcOH-BuOH (7:2)	CuSO <sub>4</sub> -NH <sub>4</sub> OH	229
	C <sub>6</sub> H <sub>6</sub> -Acetone-AcOH (4:4:2)	" "	229
TLC	Propanol-Ethyl Acetate Water-25% NH <sub>4</sub> OH (5:1:3:1)	" "	229
TLC			230
TLC	-	Ninhydrin	231
Dowex 50-TLC	1.5M NaOAc pH 8.5 + 10% t-BuOH	Ninhydrin Cd(OAc) <sub>2</sub>	232
TLC		Bioautography	233
TLC			234

### 7.14 Electrophoresis Methods

Peptides are commonly separated by electrophoretic methods. A few methods specifically designated as identity tests are listed here. Other electrophoretic systems may be found in section 7.42.

Lightbrown and DeRossi utilized agar gel electrophoresis<sup>235</sup>. Using this basic method, Bozzi and Valdebouze developed a bioautographic system for 14 antibiotics including bacitracin<sup>236</sup>. Grynne developed a paper electrophoresis-bioautographic system for a number of antibiotics including bacitracin<sup>237</sup>.

## 7.2 Microbiological Assays

### 7.21 Tube Dilution Assay

Patrick, Craig and Bachman correlated the results of serial dilution assays with those obtained by agar diffusion assays<sup>240</sup>.

### 7.22 Turbidimetric Assay

Although the agar diffusion assay technique is the primary method for bacitracin, a number of turbidimetric methods have been reported.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
<u>Staphylococcus aureus</u>	Darker, <u>et al.</u>	241
Na resazurin indicator	De Felip, <u>et al.</u>	242
<u>Streptococcus faecalis</u>	Pain, Bose, Dutta	243
Autoanalyzer method	Platt, Gentile and George	244
Na resazurin indicator	Ruffo and Socci	245
<u>Escherichia Coli</u>	Rappe, Mauquoy and Bauer	246
Zinc bacitracin in feeds	Ragheb, Black and Graham	247



Kirschbaum, Arret and Harrison published statistical procedures for determining the dose-response curve for turbidimetric assays<sup>248</sup>.

### 7.23 Agar Diffusion Assays

The majority of microbiological assays for bacitracin involve the use of agar diffusion methods. Some highlights of these methods are presented in tabular form.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
<u>Staphylococcus aureus</u> , prediffusion	Darker, <u>et al.</u>	241
Development of diagnostic discs	Patrick, Craig, Bachman	240
Effect of medium composition	Neter, Murdock, Kunz	249
<u>Corynebacterium xerosis</u>	Porath	250
Assay of bacitracin in Galenical products	Trolle-Lassen	251
<u>Micrococcus flavus</u>	Pinzelik, Nisonger Murray	252
Organisms resistant to other antibiotics	Friedman, Kirschbaum	253
Assay of emulsion formulation	Varma, Hall, Rising	254
<u>Sarcina lutea</u>	Vuilleumier, Anker	255
Diagnostic discs	Kirschbaum, Kramer, Arret	256
Disc plate assay	Rossi	257
Bacitracin in feed	Craig	258
Disc plate assay	Bauer	259
Sensitivity of method	Pitton	260

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Antibiotic mixtures	Yonezawa, <u>et al.</u>	261
Bacitracin in feed	Craig	262
Bacitracin in tissue	Freres, Valdebouze	263
Diffusion characteristics	Cluzel, Cluzel, Michel, Sirot	264
Bacitracin in milk	Read, Bradshaw, Swartzentruber	265
<u>Bacillus</u> <u>stearothermophilus</u>	Kabay	266
Bacitracin in feeds-gel filtration	Skodova, <u>et al.</u>	267
Sensitivity tablets	Casals, Gylling, Pedersen	268
Bacitracin in milk, tissue	Rybinska	269
Bacitracin in fermentation broth	Haavik	128
Use of tetrazolium dyes	Picmanova, <u>et al.</u>	270
Bacitracin in tissue	Smither	271
Bacitracin in tissue	Krystyna-Skonieczna, Rybinska	272
Bacitracin in feeds-molecular sieve	Skodova, Skarka	273
Interference	Liskova, Kohoutkova	274
Bacitracin in animal feeds	Pacini, Meneghini	275
Frozen inoculum	Hadfield	276
Bacitracin in anti-biotic mixtures	DeCarneri	277

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Vertical agar diffusion	Lameris, <u>et al.</u>	278

#### 7.24 Feed Assays

Several of the microbiological assays already mentioned can be used to assay bacitracin or zinc bacitracin in feeds. The following papers detail extraction methods which can be used to extract bacitracin from complex animal feeds.

<u>Author</u>	<u>Reference</u>
Randall	279
Randall and Burton	280
Wright and Burton	281
Craig	282
Grynne and Hoff	283
Grynne	284
Grynne, Hoff, Silsand and Vaaje	285
Fassbender and Katz	286

#### 7.25 Miscellaneous Assays

The microbiological assay of bacitracin in antibiotic mixtures, soils and body fluids has been discussed in some of the papers specified in sections 7.21 through 7.24. The following papers are of special interest in the assay of these samples:

<u>Assay Notes</u>	<u>Authors</u>	<u>Reference</u>
Bacitracin and neomycin	Lingnau and Machek	287
Bacitracin and neomycin	Balliu and Boteanu	288
Bacitracin in soil	Soulides	289

<u>Assay Notes</u>	<u>Authors</u>	<u>Reference</u>
Blood level assay	Eagle, <u>et al.</u>	290
Stool assay	Wilson, Ing, Metcalf-Gibson and Wrong	291
Animal tissue	Kline and Rathmacher	292
Bacitracin standard	Kirschbaum, Arret and Kramer	293
Review of methods	Dennin	294
Temperature of incubation	Hinks, Daneo- Moore and Braverman	295
Electrical polarization	Morris and Jennings	296

### 7.3 Chemical Methods

Although microbiological methods appear to be preferred for bacitracin, several types of chemical and biochemical assays have been proposed for the antibiotic.

#### 7.31 Gravimetric and Colorimetric

Maturana, Dannier and Brieva have proposed a gravimetric phosphotungstic acid method for bacitracin<sup>297</sup>.

Doulakas has published a colorimetric assay involving the reaction with phloroglucinol after the oxidation of the antibiotic with hypobromite<sup>298</sup>.

#### 7.32 Electrochemical Assays

Caplis, Ragheb and Schall have proposed an alternating current polarographic assay for bacitracin<sup>299</sup>.

Skarka and Sestakova have reported a oscillopolarographic method as well as a

sensitive colorimetric method<sup>300</sup>.

Jacobsen, Pederstad and Oeystese have utilized differential pulse polarography to assay bacitracin and zinc bacitracin<sup>301</sup>. The degradation product, bacitracin F, is reduced at a less negative potential.

### 7.33 Determination of Zinc in Zinc Bacitracin

Charles and Weiss utilized an EDTA titration to measure the concentration of zinc in zinc bacitracin<sup>302</sup>.

More recently, atomic absorption spectroscopy has been utilized for this assay<sup>303, 304</sup>.

### 7.34 Biochemical Assays

As is the case with other antibiotics, investigators have established that certain enzyme systems are inhibited by the presence of bacitracin. In general, these methods have not been shown to be as useful as microbiological assays but we have included a few references which may be of general interest.

<u>Enzyme System</u>	<u>Author</u>	<u>Reference</u>
D-Amino acid oxidase	Hayashi	305
Arginine diaminase	Mikolajcik	306
Proteolytic enzymes	Coppi and Bonardi	307
Pancreatic lipase	Coppi and Bonardi	308
Human spermatozoa	Schirren	309

## 7.4 Chromatographic Methods

### 7.41 Countercurrent Distribution

At the time when bacitracin was discovered, countercurrent distribution was probably the most popular separation technique. Although it has been supplanted by various types of

chromatography on solid supports it is still useful for the separation of large molecules such as the bacitracins.

<u>System Notes</u>	<u>Authors</u>	<u>Reference</u>
Separation of bacitracin in one major and two minor fractions	Barry, Gregory and Craig	12
Separation into more than one component	Craig	310
Amyl alcohol-butanol-pH 7.0 buffer	Newton and Abraham	6
Isolated pure A, B and C	Newton and co-workers	311
Separation into 1 major and 4 minor components	Craig and co-workers	312
Separation into 10 components	Newton and Abraham	13
CHCl <sub>3</sub> -methanol-water (2:2:1)	Konigsberg and Craig	313
MeOH-H <sub>2</sub> O-C <sub>6</sub> H <sub>6</sub> -CHCl <sub>3</sub> (23:7:15:15)	Ramachandran	314
Separation of commercial bacitracin into 10 components	Hausmann, Weisiger and Craig	315
A number of systems utilized	Craig and Konigsberg	35
Countercurrent dist. of DNP derivative	Craig, Hausmann and Weisiger	316
Separation of bacitracin A into two isomers	Craig, King and Konigsberg	317
BuOH-C <sub>5</sub> H <sub>5</sub> N-AcOH-H <sub>2</sub> O (20:5:5:30) separation of degradation products	Konigsberg, Hill and Craig	38

<u>System Notes</u>	<u>Authors</u>	<u>Reference</u>
30% Ethyl acetate-70% 1 butanol-pH 5.43 buffer	Craig and co-workers	91

#### 7.42 Electrophoresis

Electrophoresis on a variety of substances has been utilized frequently in the separation of large molecular weight molecules possessing an ionic charge.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Starch column	Flodin and Porath	318
Cellulose column	Porath	319
Paper electrophoresis	Proenca da Cunha and Baptista	320
Paper electrophoresis	Paris and Theallet	321
Paper electrophoresis	Apreotesei and Teodosiu	322
Paper electrophoresis	Proenca da Cunha and Gomes	323
Paper electrophoresis	Pirila, Saukkonen and Santaoja	197
Agar gel	Swank and Munkres	325
Paper electrophoresis	Maeda, Yagi, Naganawa, Kondo and Umezawa	324
Polyacrylamide gel	Swank and Munkres	325
Agar gel	Dubost and Pascal	326
Polyacrylamide gel	Coombe	327

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Low voltage	Langner and co-workers	328
Electrophoresis of feed and foods	Langner	329
Gelatin gel	Bozzi and Valdebouze	236
Identification test	Grynne	237
Isoelectric focusing in gel	Froeyshov	330

#### 7.43 Column Chromatography

Column chromatography utilizing a variety of support materials has been used to perform crude separations of bacitracin fractions.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Charcoal-celite column	Porath	250
Charcoal-celite (1:3)-0.1N acetic acid	Porath	319
Carboxymethylcellulose	Konigsberg and Craig	89
Carboxymethylcellulose	Konigsberg, Hill and Craig	38
Carboxymethylcellulose	Storm and Strominger	331

#### 7.44 Gel Filtration

Gel filtration has been extensively used to separate macro molecules on the basis of molecular size. Bacitracin has been utilized as a standard in several systems since it is well characterized.



<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Sephadex G-25 (Propanol-acetic acid- water)	P.R. Carnegie	332
Sephadex G-10 (acetic acid-NaCl)	Eaker and Porath	333
Sephadex G-100	Reickert and co-workers	334
Sephadex LH-20	Gregerman, Weaver and Kowatch	335
Agarose	Bryce and Crichton	336
Polyethyleneglycol dimethacrylate gel	Randau, Bayer and Schnell	337
Sephadex G-25, G-50	Catsimpoolas and Kenny	338
Polyacrylamide gel	Stewart	339
Bio-Gel P-2 (tissues)	Skarka, Skodova and Skoda	340

#### 7.45 Paper Chromatography

Paper chromatography is frequently used for the separation of antibiotics because the components can conveniently be located by bioautography.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Bioautography of various antibiotics	Snell, Ijichi and Lewis	228
Ninhydrin pyridine- acetic acid	Castel, Mus and Storck	341
Butanol-acetic acid- water (50:25:25)	daCunha and Baptista	342

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
"Salting out" chromatography	daCunha and Baptista	343
Three solvent systems	Paris and Theallet	321
Dyes as detection reagents	Singh	344
Hydrophobic system	Ritschel and Lercher	345
Det. of Bacitracin in fodder	Louis	346
Separation of 42 antibiotics	Schmitt and Mathis	347

#### 7.46 Thin Layer Chromatography

Thin layer chromatography is also widely used for the chromatography of antibiotics because of its rapidity.

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Silica gel and kieselgel	Paris and Theallet	321
Silica gel ethanol, $\text{NH}_4\text{OH}-\text{H}_2\text{O}$ (8:1:1)	Umezawa and co-workers	348
Silica gel ethanol-water (4:1)	Akita and Ikekawa	349
Butanol-acetic acid-water (3:1:1)	Umezawa and co-workers	350
Separates Bacitracins A and F	Nussbaumer	351
Separates various antibiotics	Pitton	352
	McGilveray and Strickland	353
$\text{CuSO}_4$ color reaction	Guven and Ozsari	229

<u>Method Notes</u>	<u>Authors</u>	<u>Reference</u>
Identification of sensitivity discs	Wayland and Weiss	230
Bioautography	Aszalos, Davis and Frost	354
	Fooks, McGilveray and Strickland	355
	Reimers	356
5 Solvent systems	Stretton, Carr, Watson-Walker	357
Butanol-H <sub>2</sub> O-pyridine-AcOH-ethanol	Carr, Stretton and Watson-Walker	358
Dowex-50 plates	Pauncz	359
Resin coated plates	Pauncz	232
Detection of anti-biotics in meat	Langner and Tuefel	231
Bioautography	Langner and Teufel	328
Cellulose plates	Langner and Tuefel	329
Determination in feed	Freres and Valdebouze	233
Determination in tissue	Baldini and co-workers	360
Determination in milk	Bossuyt and co-workers	234

#### 7.47 High Pressure Liquid Chromatography

High pressure liquid chromatography is one of the newest chromatographic methods. The technique combines a high resolution column with a detector, so the method is generally not only selective but precise.

Spechter has utilized a silica

column coated with Carbowax 20M<sup>361</sup>.

Tsuji, Robertson and Bach used Bondapak C<sub>18</sub>/Corasil with gradient elution to separate the components of bacitracin<sup>362</sup>.

Tsuji and Robertson improved on the previous method by using a micro-Bondapak C<sub>18</sub> column<sup>196</sup>.

Dr. Yeh adopted the general method of Tsuji and Robertson<sup>196</sup> for the examination of some samples of commercial bacitracin obtained by our laboratory<sup>407</sup>. (Samples of bacitracin and zinc bacitracin were generously supplied by International Minerals and Chemicals Corporation and by A/S Dumex Ltd. In addition, the U.S.P. Standard of zinc bacitracin was chromatographed).

Although the column and solvent system employed by Dr. Yeh were the same as those reported by Tsuji and Robertson, he was unable to reproduce the exact exponential gradient they utilized because of equipment limitations. As a result, the peaks were not as sharp and he was unable to obtain separation of some of the components. The major component in all the samples appeared to be bacitracin A. A component eluting just before bacitracin A was probably bacitracin B<sub>1</sub> or B<sub>2</sub>. The other components appeared to be present in much smaller concentrations. In all samples, eight to ten components could be seen.

Dr. Yeh experienced some base line drift because of the change in gradient composition. We were gratified with the separation that Yeh was able to achieve with the limited amount of time he was able to devote to the project.

## 8. Mode of Action

Gale found that, like other antibiotics, bacitracin interfered with protein biosynthesis<sup>363</sup>. Gale and Folkes studied the inhibition of incorporation of amino acids into proteins using a cell homogenate<sup>364</sup>.

Schechter, Momose and Rudney found that

bacitracin interfered with biosynthetic pathways which involved polyprenylpyrophosphates <sup>365</sup>.

Storm and Strominger found that bacitracin interacted with C55 isoprenylpyrophosphate in the cell membrane<sup>366</sup>. This altered the permeability of the bacterial cell.

#### 9. Derivatives of Bacitracin

A number of bacitracin derivatives have been produced. Some of these have been suggested for use in animal feeds.

Siminoff, Price and Bywater suggested that the methylene disalicylic acid complex of bacitracin was useful as a feed additive for swine and poultry <sup>367</sup>. Radomski, Hagan, Nelson and Welch established the toxicity and safety of this derivative<sup>368</sup>. This complex was approved as a feed additive<sup>369</sup>.

Manganese bacitracin is an approved feed additive<sup>370</sup>.

A Japanese patent was issued for the sodium methanesulfate derivative of bacitracin<sup>371</sup>.

A U.S. patent was issued to Lewis, Ninger and Pattison for the synthesis of the sodium methane-sulfonate derivative of bacitracin which they suggested was suitable for parenteral administration <sup>372</sup>. Baldwin patented sodium, potassium, calcium, zinc and manganese salts of bacitracin methane-sulfonate<sup>373</sup>.

SPOFA United Pharmaceutical Works reacted bacitracin with a number of aldehydes and then isolated the corresponding zinc salts<sup>374</sup>. Vondracek, Toscaniova and Hoffman have patented a furfural derivative of bacitracin<sup>375</sup>.

Kalina, Ulbert and Masita patented the diisobutyl-naphthalenesulfonate derivative of bacitracin <sup>376</sup>.

Atassi and Rosenthal reduced bacitracin with diborane<sup>377</sup>. Shipchandler was issued a patent on derivatives of bacitracin reduced with sodium borohydride<sup>378</sup>.

Mancino, Tigelaar and Ovary compared the antigenic properties of the three monodinitro-phenyl derivatives of bacitracin with that of the tri-dinitrophenyl derivative<sup>379</sup>.

A Japanese patent was issued in which bacitracin was reacted with polyamine ion exchange resins by means of an aldehyde<sup>380</sup>. The resulting product was insoluble. In the same way, a dimer of bacitracin was produced by reacting the antibiotic with glyoxal<sup>381</sup>.

#### 10. Reviews

Two reviews have been published on the assay of bacitracin<sup>193,382</sup>.

A number of reviews have been published on bacitracin<sup>383,384,385,386, 387, 388, 98, 389, 390, 391</sup>.

Many more reviews have included bacitracin along with other antibiotics <sup>392,393,394,395,396, 397,398,399,400,401,402,403, 404,405,406</sup>.

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Literature search complete through 1978.

# BRETYLIUM TOSYLATE

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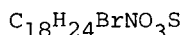
## 1. Description

### 1.1 Chemical and Proprietary Names

Bretylium tosylate is the non-proprietary name for o-bromobenzylethyldimethylammonium p-toluenesulfonate. It has been marketed as an antihypertensive agent but is no longer used for this indication in the United States. Proprietary names listed by the Merck Index are Bretylan, Bretylate, Darenthin and Ornid.

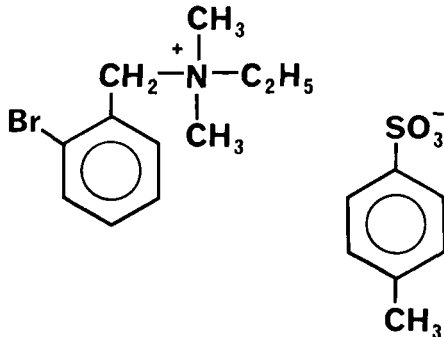
The drug is now marketed as an antiarrhythmic agent with the proprietary name Bretylol.

### 1.2 Empirical Formula



Molecular Weight 414.36 The Merck Index (1) lists the molecular weight as 414.39. Based upon atomic weights defined in 1973 by the International Union of Pure and Applied Chemistry 414.36 is correct.

### Structure



### 1.3 Appearance, Color, Odor and Taste

Bretylium tosylate is a white to off-white free flowing, fine, odorless powder. It has an extremely bitter taste.

## 2. Physical Properties

### 2.1 Melting Range

96°C - 99°C

## 2.2 Solubility Profile

Bretylium tosylate is freely soluble in water, methanol and ethanol. It is commonly recrystallized from hot acetone. Chloroform and methylene chloride are the best extraction solvents. Bretylium tosylate is essentially insoluble in ether, ethylacetate and hexane.

## 2.3 Infrared Spectrum

The KBr pellet infrared spectrum of 0.5% bretylium tosylate obtained with a Perkin-Elmer 283 Infrared Spectrophotometer is contained in Figure 1. Bretylium tosylate is very hygroscopic. Unless the spectrum is obtained on dried material the broad O-H stretching band centered at  $3460\text{ cm}^{-1}$  will be present. The aromatic ( $3100 - 3000\text{ cm}^{-1}$ ) and aliphatic ( $3000 - 2900\text{ cm}^{-1}$ ) C-H stretching bands are present but not as strong as might be anticipated. The strong broad peak centered at  $1200\text{ cm}^{-1}$  is the S-O stretching band. The molecule contains both a para substituted aromatic (strong C-H bending at  $815\text{ cm}^{-1}$ ) and an ortho substituted aromatic (strong C-H bending at  $772\text{ cm}^{-1}$ ) ring.

For routine identification purposes a liquid infrared spectrum is generally more reproducible. The spectrum of a 2% solution in dry chloroform is shown in Figure 2.

## 2.4 Ultraviolet Spectrum

Bretylium tosylate absorbs strongly in the ultraviolet region of the spectrum with three distinct maxima between 230 nm and 300 nm. The spectrum (Figure 3) was obtained with a Beckman Acta III double beam spectrophotometer. The wavelength maxima and molar absorptivities are:

<u><math>\lambda</math> (nm)</u>	<u><math>\epsilon</math><sub>1</sub></u>
278	671
271	885
264	886
257 (shoulder)	---

## 2.5 Proton Magnetic Resonance Spectrum

The 60 MHz proton magnetic resonance spectrum was obtained with a Varian Associates T-60A spectrometer. The spectrum in  $\text{CDCl}_3$  with tetramethylsilane (TMS) as internal reference is contained in Figure 4. The integration and

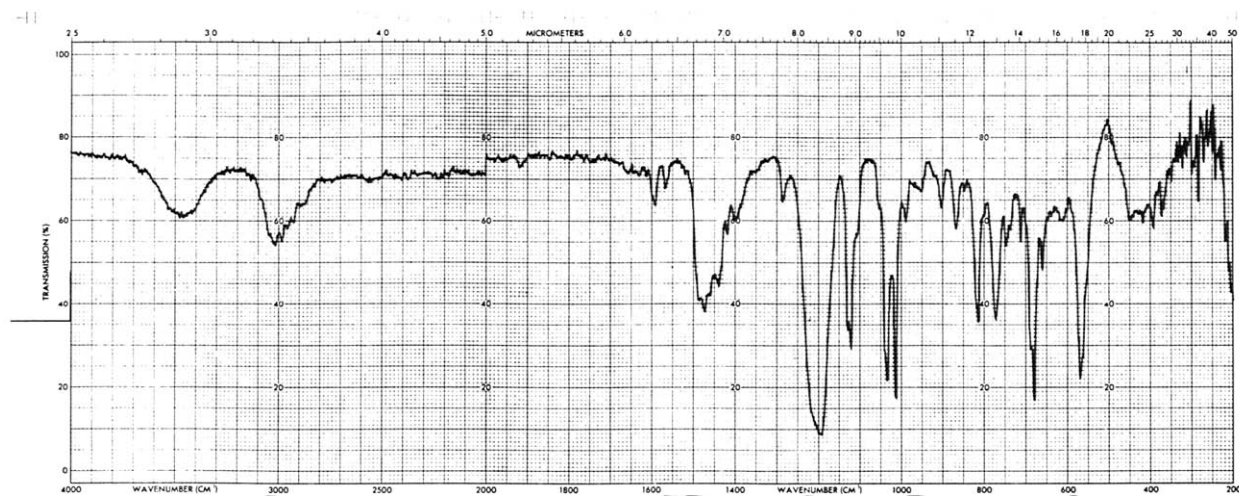


Figure 1. KBr Infrared Spectrum of Bretylium Tosylate.

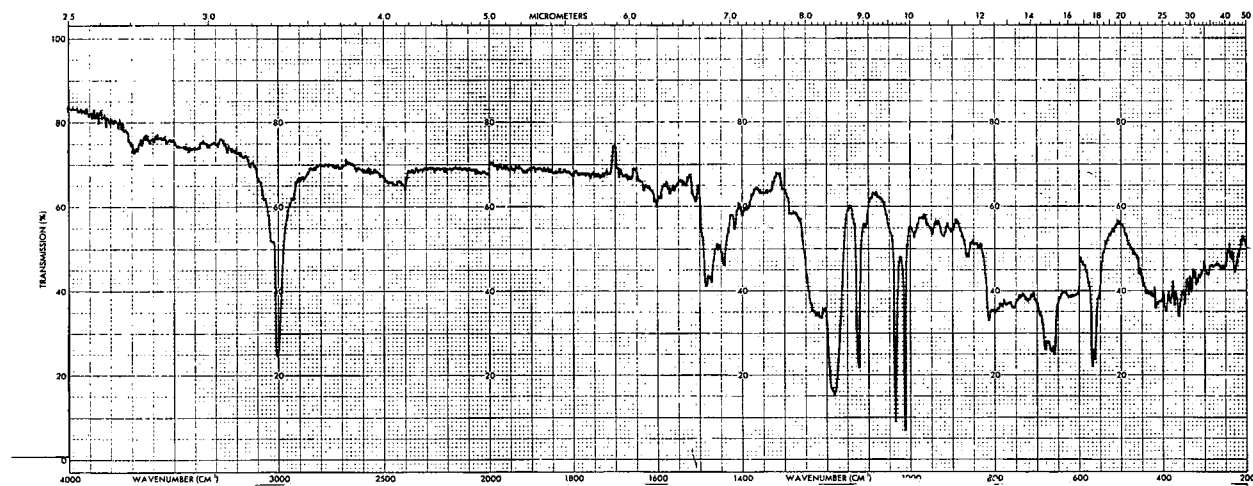


Figure 2. Infrared Spectrum of 2% Bretylium Tosylate in Dry Chloroform.

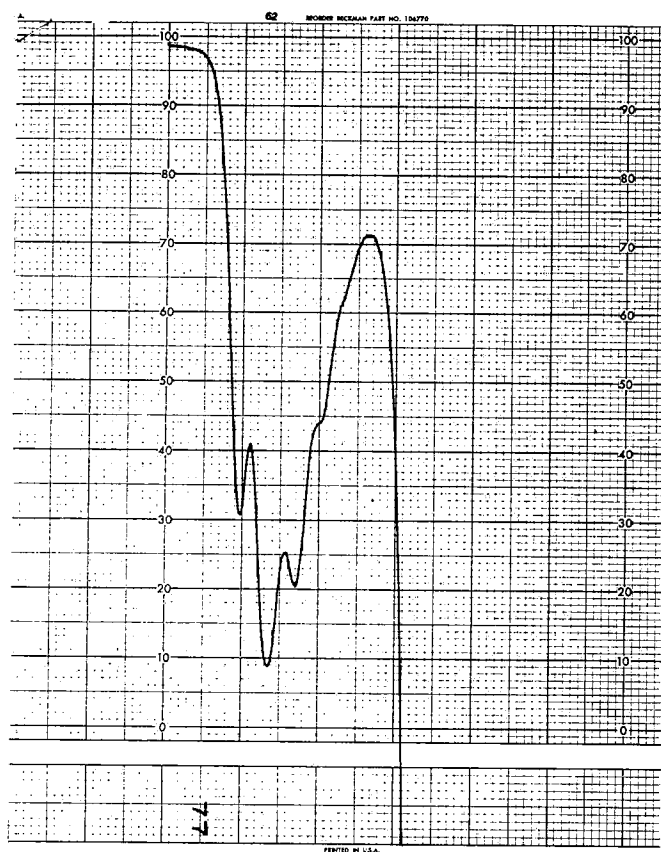


Figure 3. Ultraviolet Spectrum of Bretylium Tosylate.

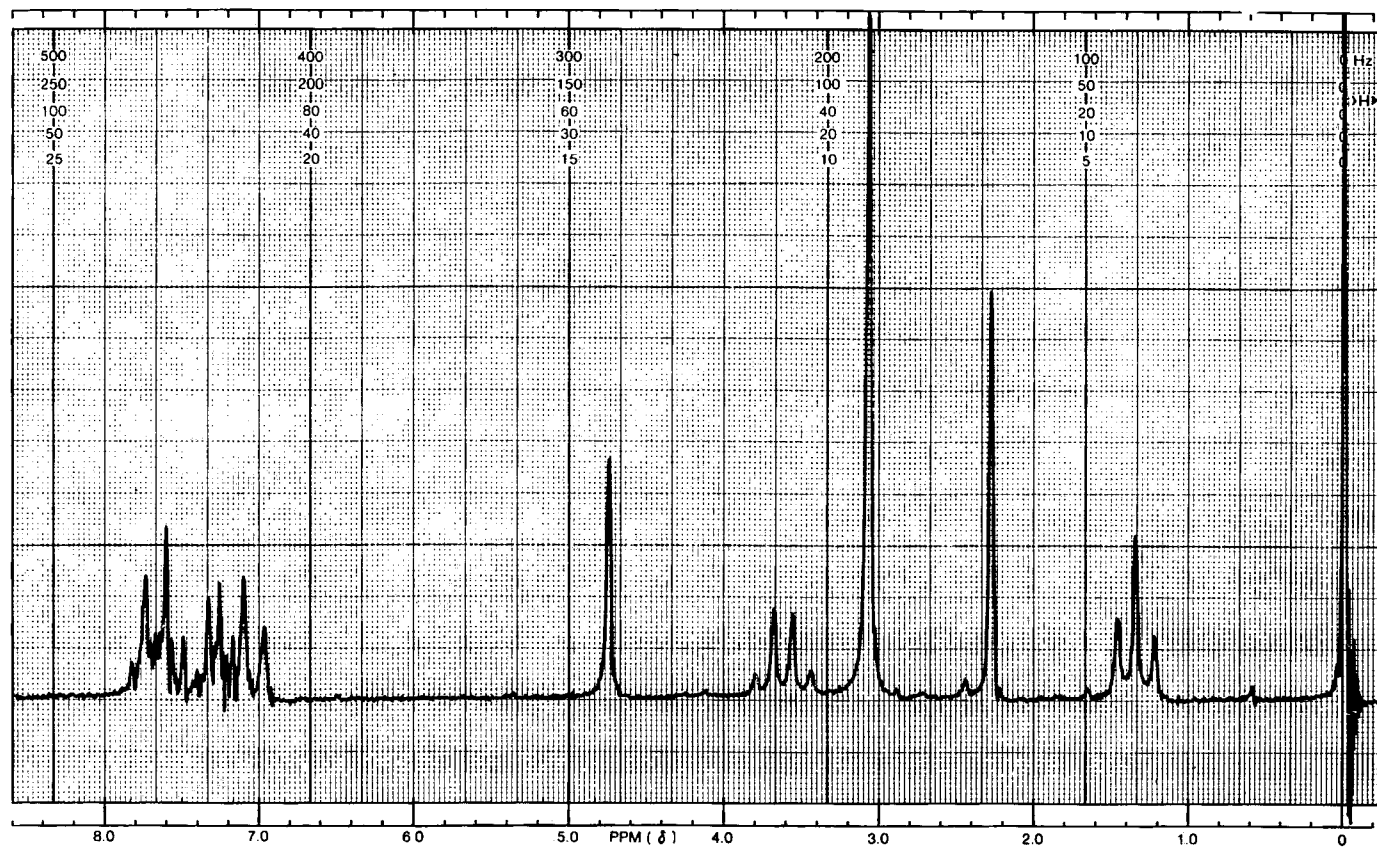
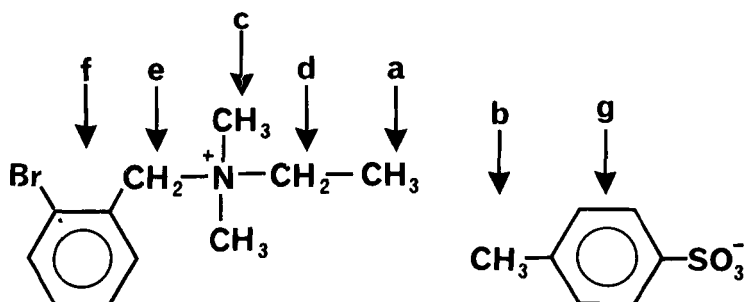


Figure 4. Proton Magnetic Resonance Spectrum of Bretylum Tosylate in CDCl<sub>3</sub>.

multiplicities are consistent with the proton assignments.

Chemical shifts ( $\delta$ ) in ppm relative to TMS are:



<u>Proton Assignment</u>	<u># of Protons</u>	<u>Chemical Shift (<math>\delta</math>)</u>	<u>Multiplicity</u>
a	3	1.35	triplet
b	3	2.27	singlet
c	6	3.07	singlet
d	2	3.65	quartet
e	2	4.73	singlet
f	4	7.17	multiplet
g	4	7.67	multiplet

## 2.6 Mass Spectrum

The direct probe electron impact mass spectrum of bretylium tosylate is shown in Figure 5. The spectrum was obtained with a Dupont Dimaspec GC/MS Model 321 (2). No parent ion is seen because bretylium tosylate is a salt and will not travel through the spectrometer intact. Principal fragment ions in the spectrum are identifiable. The base peak at  $m/z$  91 is the tropylium ion ( $C_7H_7^+$ ) probably formed by loss of  $SO_3^-$  from tosylate. The tropylium ion is also possible from fragmentation of the bretylium ion. The  $m/z$  58 is  $C_3H_8N^+$  formed by loss of  $C_2H_5$  (which is possible by a number of different paths) from the bretylium quaternary ammonium side chain. The two isotopes of bromine of mass 79 and 81 make the peaks at  $m/z$  169, 171 and  $m/z$  185, 187 readily identifiable as  $C_7H_6 Br^+$  and  $C_7H_8N Br^+$  respectively.

## 2.7 Differential Scanning Calorimetry

Bretylium tosylate was heated at a rate of  $20^\circ/\text{min}$  in a Perkin-Elmer Model DSC-2 differential scanning calori-

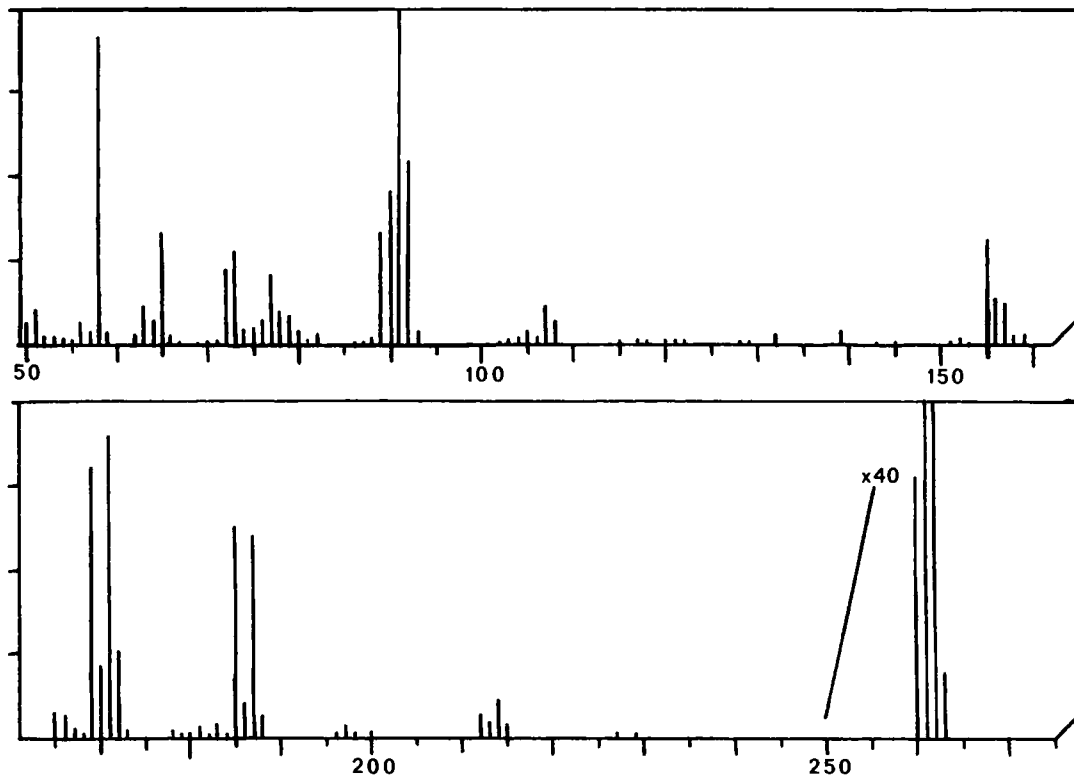


Figure 5. Electron Impact Mass Spectrum of Bretylium Tosylate.



meter. A single endotherm was observed with an onset temperature of  $97.5^{\circ}\text{C}$  with the endotherm maximum at  $102.5^{\circ}\text{C}$ . The onset temperature corresponds to the melting point. The heat of transition ( $H$ ) calculated in relation to an indium standard is 16.8 cal/g.

## 2.8 Crystal Properties

Bretylium tosylate crystals examined with a polarizing microscope were found to be tetragonal prisms, elongated parallel to the  $c$  crystallographic axis (3). X-ray diffraction patterns (Table I) were also determined (3).

Table I. Powder x-ray diffraction pattern of Bretylium Tosylate

$2\theta$	Relative Intensity	$d(\text{\AA})$
7.65	50	11.6
11.05	50	8.00
12.65	10	6.99
14.10	25	6.28
15.25	30	5.81
16.60	10	5.34
18.00	5	4.92
18.65	5	4.75
19.35	100	4.58
20.10	5	4.41
21.30	15	4.17
22.15	50	4.01
23.10	75	3.85
24.35	10	3.65
24.75	100	3.60
26.45	80	3.37

## 3. Synthesis

The Bretylium Unites States patent contains examples for the synthesis of numerous bretylium salts (4).

## 4. Analysis

### 4.1 Elemental Analysis

Elemental analysis of a typical bretylium tosylate

sample is as follows:

<u>Element</u>	<u>% Theoretical*</u>	<u>% Found**</u>
C	52.18	52.40
H	5.84	5.76
Br	19.28	19.56
N	3.38	3.28
O	11.58	11.69
S	7.74	----

\*Calculated for  $C_{18}H_{24}BrNO_3S$

\*\*Determined on a dried sample

#### 4.2 Non-aqueous Titration

Bretylium tosylate may be measured by non-aqueous titration with 0.025 N perchloric acid in dioxane. The end point is visually detected by a change from violet to blue-green using crystal violet as the indicator.

#### 4.3 High Performance Liquid Chromatography (HPLC)

Two reversed phase HPLC methods have been developed for the quantitation of bretylium tosylate. In the first method (5) bretylium and tosylate ions are determined simultaneously with benzenesulfonic acid as an internal standard. Chromatography is carried out on a 10  $\mu$ m octadecylsilane column with an isocratic mobile phase consisting of 30% methanol in water (pH 5.0) containing a paired ion reagent, tetrabutylammonium phosphate. Flow rate through the column was 2.0 ml/min and the variable wavelength UV detector was set at 220 nm. Standards containing from 0.1 to 0.5 mg bretylium tosylate per ml of solution were employed. The method is applicable for raw drug evaluations, analysis of intravenous solutions and compatibility studies with other drugs. It is not amenable to determination of bretylium or tosylate in biological fluids. Total analysis time is less than 12 minutes.

The second reversed phase HPLC method was employed for the quantitation of bretylium ion (6). Bretylium tosylate standard concentrations ranged from 10 to 400  $\mu$ g/ml; the internal standard was the 2,4-dichloro congener of bretylium tosylate. The 30 cm by 3.9 mm column was packed with 10  $\mu$ m alkyl nitrile bonded silica. The compounds were eluted with a mobile phase consisting of acetonitrile and 0.005 M sodium phosphate monobasic in purified water (30:70) at a flow rate of 2.0 ml/min. A fixed wavelength UV detector at 254 nm was

used to monitor the column effluent. As with the first HPLC method this method is only applicable to evaluation of the raw material and dosage forms. Total analysis time by this method is approximately 15 minutes.

Both methods are specific, accurate, rapid and precise.

#### 4.4 Gas-Liquid Chromatography (GLC)

A quantitative, stability indicating GLC assay for bretylium tosylate is also applicable to dosage forms (7).

The method is based upon a published assay for estimating plasma and urine levels of the drug (8). p-Chlorobenzyl-ethyldimethylammonium p-toluene sulfonate (bretylium is the o-bromo congener) was synthesized and used as the internal standard. The method has been used to evaluate the stability of raw material, tablets and injections. The identity of the peaks appearing in the chromatogram has been confirmed by GLC-mass spectrometry. The method involves reaction of bretylium and internal standard with sodium thiophenolate at 70° for 15 minutes. The resultant halogenated benzylthioethers are quantitated by GLC. The reaction is specific for quaternary amines.

Chromatography was performed with a suitable gas chromatograph equipped with a flame ionization detector. The 1.8 m by 4 mm id glass column was packed with 3% OV 225 on 100-120 mesh Chromosorb W-HP. The column and inlet temperatures were maintained at 210° while the detector temperature was 275°. The carrier gas was helium at a flow rate of 50 ml/min.

The reaction scheme for bretylium and internal standard with sodium thiophenolate is shown in Figure 6. Three peaks appear in the chromatogram following the solvent front. These were identified by GLC-mass spectrometry (MS) with a DuPont DP-1 system in the electron impact (EI) mode (9). Diphenyldithiol appears at 3.0 min; EIMS, 218 ( $M^+$ ). p-Chlorobenzylphenylthioether appears at 4.0 min; EIMS, 234 ( $M^+$ ) while o-bromobenzylphenylthioether appears at 4.8 min; EIMS 278 ( $M^+$ ) and 280 ( $M^+$ ).

All EIMS spectra showed the base peak at 110 corresponding to the  $C_6H_5SH^+$  ion.

The 15 minute reaction time and 6 minute analysis time is much more practical than the 1 hour reaction time and 20 minute analysis time reported previously (8).

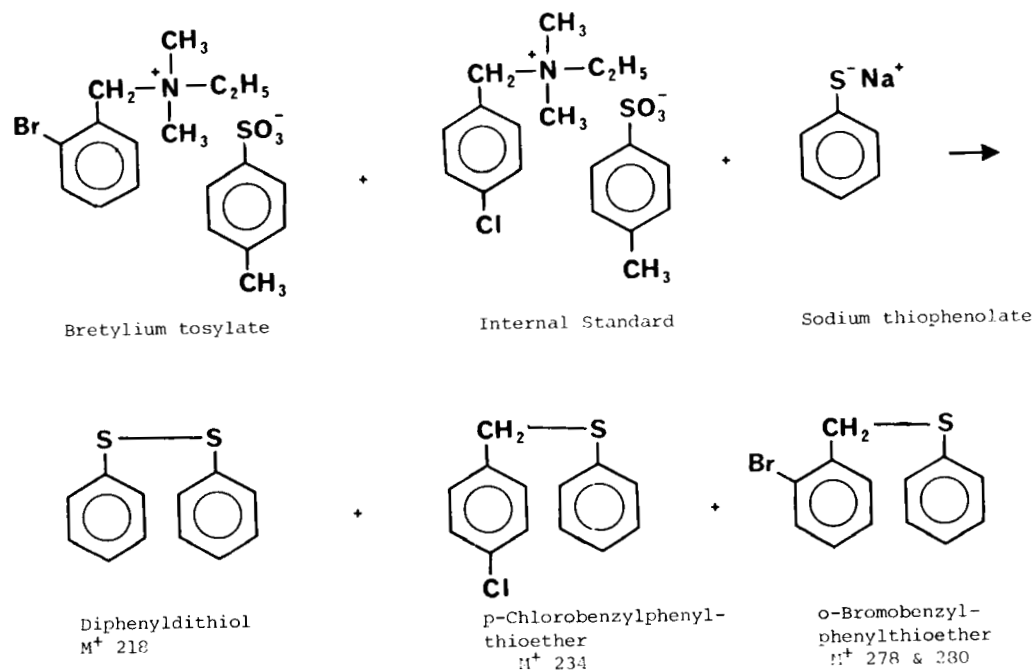


Figure 6. Reaction Scheme Showing Products Identified by GLC-Mass Spectrometry.

#### 4.5 Thin-layer Chromatography (TLC)

Purity and stability of the raw drug have also been assessed by thin-layer chromatography. Bretylium tosylate has an  $R_f$  of 0.50 when chromatographed on Alumina-G with 1-butanol saturated with water as solvent. *o*-Bromobenzyldimethyl amine is the most likely contaminant and degradation product. When the plate is sprayed with modified Dragendorff's reagent it appears as a pink spot with an  $R_f$  of 0.85.

#### 5. Stability

Bretylium tosylate is a very stable molecule. Solutions of bretylium tosylate at 50 mg/ml in 1 *N* hydrochloric acid, 1 *N* sodium hydroxide and 10% hydrogen peroxide were heated for one hour at 90°C. The solutions were analyzed by the stability indicating gas chromatographic (section 4.4) and thin-layer chromatographic (section 4.5) methods. The ultraviolet absorbance at 271 nm was also monitored.

The results (Table II) indicate the potency of the solutions did not change with this drastic treatment.

Table II. Subjection of bretylium tosylate to drastic acid, alkaline and oxidative conditions.

<u>Solution</u>	<u>Assay*</u>	<u>Initial Assay</u>	<u>Final Assay</u>	<u>Change From Initial</u>
1 <i>N</i> HCl	GLC	100.0%	100.5%	+0.5%
	TLC	one spot**	one spot	no change
	UV	0.824	0.832	+1.0%
1 <i>N</i> NaOH	GLC	101.5%	100.5%	-1.0%
	TLC	one spot	one spot	no change
	UV	0.825	0.838	+1.6%
10% H <sub>2</sub> O <sub>2</sub>	GLC	101.5%	102.9%	+1.4%
	TLC	one spot	one spot	no change
	UV	0.835	0.821	-1.7%

\*GLC - gas-liquid chromatography

TLC - thin-layer chromatography

UV - ultraviolet absorbance at 271 nm.

\*\*one spot indicates one spot with an  $R_f$  matching bretylium tosylate standard.

#### 6. Analysis of Biological Samples by Gas-Liquid Chromatography

A quantitative method for the analysis of low concentrations of bretylium in plasma and urine has only recently been developed (10). The method is based upon derivatization as are the previously described GLC procedures (7,8). To enhance the sensitivity of the assay an electron capture detector was employed and 2,4,5 trichloro sodium thiophenolate was substituted for sodium thiophenolate. Internal standards employed were p-bromobenzylethyldimethylammonium p-toluenesulfonate or o-methoxybenzylethyldimethylammonium p-toluene sulfonate. Bretylium tosylate was quantitatively extracted with methylene chloride after deproteinization with acetonitrile.

The sensitivity of the method is 5 ng/ml.

Analysis was performed with a gas chromatograph and a  $^{63}\text{Ni}$  electron capture detector. The 1.8 m by 4 mm id glass column was packed with 3% OV 225 on 100/120 Supelcoport. Injection port, column and detector temperatures were maintained isothermally at 270°, 250° and 300°, respectively. Argon/methane (95/5) was the carrier gas at a flow rate of 50 ml/min (30 ml/min through the column and 20 ml/min directly to the detector as a scavenger gas).

The retention times of the 2,4,5 trichlorophenylthioether derivatives of the o-methoxybretylium congener, bretylium and the p-bromobretylium congener were 6.1, 7.4 and 9.4 min respectively.

#### 7. Absorption, Metabolism and Excretion

Bretylium is not absorbed from the stomach and is poorly absorbed from the gastrointestinal tract (11). Radioactive tracer studies indicate that the drug is not metabolized and is excreted primarily in the urine (8,12). After an intramuscular administration of  $^{14}\text{C}$  bretylium 63% of the dose was recovered in the urine and 31% was in the feces in the following 4 days (8). Bretylium was found in high concentration in the bile which suggests bile as the source of bretylium found in the feces.

The pharmacological and biochemical properties of bretylium have been reviewed (13).

#### 8. Acknowledgement

The manuscript was expertly typed by Ms. Deborah Canfield.

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# CARBAMAZEPINE

*Hassan Y. Aboul-Enein and A. A. Al-Badr*

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## CARBAMAZEPINE

1. Description1.1 Nomenclature1.11 Chemical names

5H-Dibenz [b,f] azepine-5-carboxamide  
5-Carbamoyl-5H-dibenz [b,f] azepine  
2,3 : 6,7-Dibenzazepine-1-carboxylic acid, amide

1.12 Generic Name

Carbamazepine

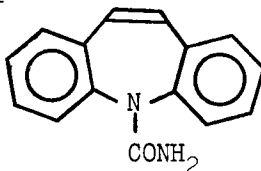
1.3 Trade names

Finlepsin, Tegretol, Tegretal

1.2 Formulae

1.21 Empirical  $C_{15}H_{12}N_2O$

1.22 Structural



1.23 Wiswesser Line Notation : TC 676 BNJ BVZ (1)

1.3 Molecular weight 236.26

1.4 Elemental composition

C 76.25%, H 5.12%, N 11.86%, O 6.77%

1.5 Appearance

White to off-white powder.

2. Physical properties

2.1 Melting point

Melts within a range of 3° between 187 and 193° (2).

## 2.2 Solubility

Practically insoluble in water; soluble in alcohol, acetone and propylene glycol (3).

## 2.3 Identification

### 2.31 Infrared Spectroscopic test

USP XIX (4) cites the use of infrared absorption spectrum of carbamazepine in methylene chloride as a mean of identification comparing some characteristic absorption bands of the drug. This will be discussed in the infrared spectral properties of the drug.

### 2.32 Color test

Carbamazepine can be identified (5) by color test with ammonium molybdate. A faint to blue color is produced (sensitivity 1.0  $\mu$ g).

BP 1973 (6) describes a color test in which 0.1 g of the drug is treated with 2 ml nitric acid in a water-bath for three minutes where an orange color is produced.

### 2.33 Crystal test

Carbamazepine can be identified by forming crystals with lead iodide solution where needles are formed (5).

## 2.4 Spectral properties

### 2.41 Ultraviolet spectrum

Carbamazepine in neutral methanol solution shows maxima at 212 nm, an inflection at 236 nm, 283 nm; and a minimum at 256 nm. (Fig. 1).

Carbamazepine (5) in ethanol shows a maxima at 215 nm and at 285 nm, minimum at about 257 nm. In 0.1 N sulphuric acid, the drug shows maxima at 283 nm (E 1%, 1 cm 147) and an inflection at about 255 nm (E 1%, 1 cm 274).

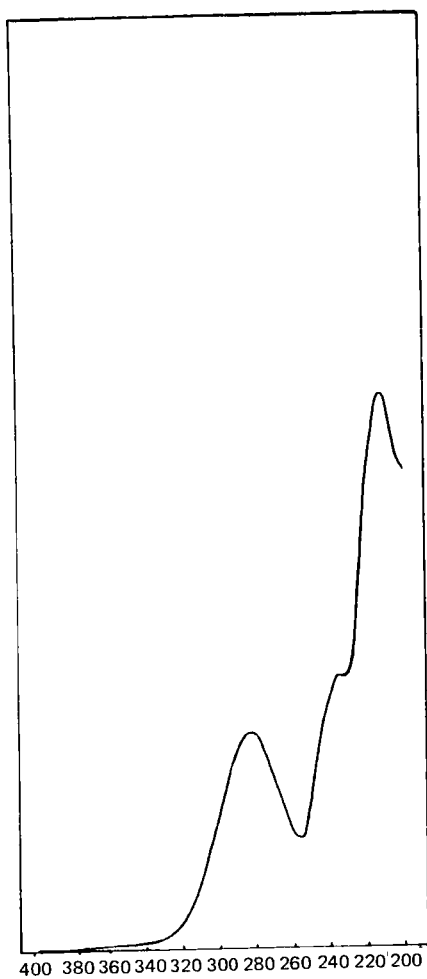


Fig. 1 - Ultraviolet spectrum of carbamazepine in methanol.

The ultraviolet absorption spectrum of the drug is used as a mean of identification of carbamazepine in BP 1973 (6). A 2 cm layer of 0.001 w/v solution in alcohol (95%) exhibits a maximum only at 285 nm; extinction at 285 nm, about 0.98.

The drug also exhibits an intense blue fluorescence in the ultraviolet light at 366 nm.

#### 2.42 Infrared spectrum

The infrared spectrum of carbamazepine is shown (Fig. 2). The spectrum was obtained from nujol mull. The structural assignments have been correlated with the following band frequencies:-

Frequency ( $\text{Cm}^{-1}$ )	Assignments
3470	$\text{NH}_2$
1680	$\text{C}=\text{O}$
1600 shoulder and 1590	Aromatic $\text{C}=\text{C}$

Clarke (5) cited the following bands as characteristic principal peaks for carbamazepine when determined in potassium bromide; 1678, 1388 and  $1594 \text{ Cm}^{-1}$ .

#### 2.43 Nuclear Magnetic Resonance Spectrum

A typical NMR spectrum of carbamazepine is shown in (Fig. 3). The sample was dissolved in  $\text{CDCl}_3$ . The Spectrum was determined on a Varian T-60A, <sup>3</sup>NMR spectrometer with TMS as the internal standard. The following structural assignments have been made for (Fig. 3).

Chemical Shift ( $\delta$ )	Assignments
Broad singlet at 4.83	$\text{NH}_2$
Singlet at 6.87	$\text{CH}=\text{CH}$ at $\text{C}_{10}^2\text{C}_{11}$
Multiplet centered at 7.33	Eight aromatic protons on the two phenyl groups.

#### 2.44 Mass spectrum and fragmentometry

The mass spectrum of carbamazepine obtained by

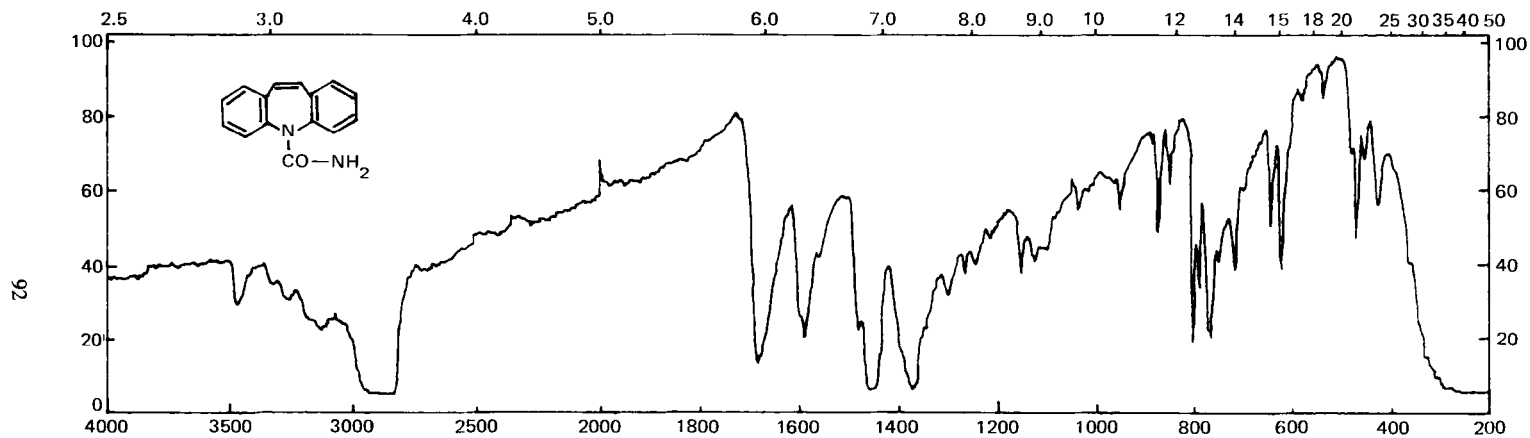


Fig. 2 - Infrared spectrum of carbamazepine in Nujol mull.

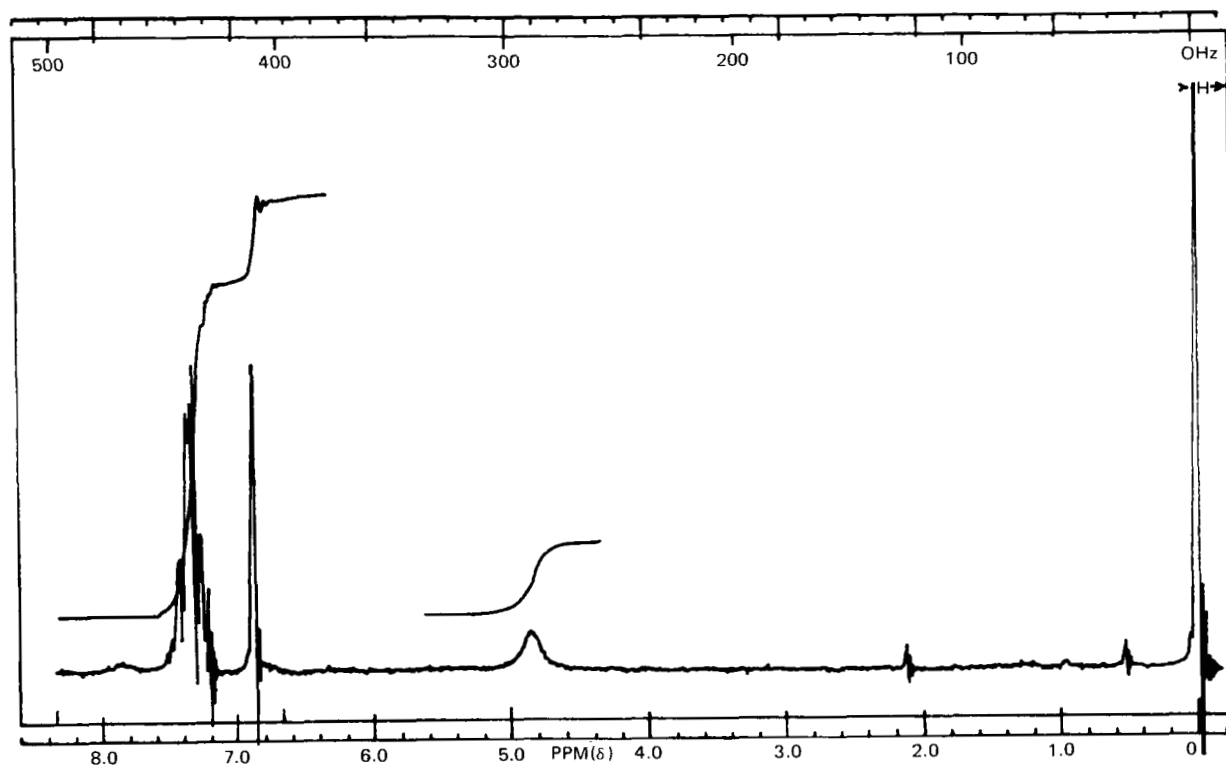
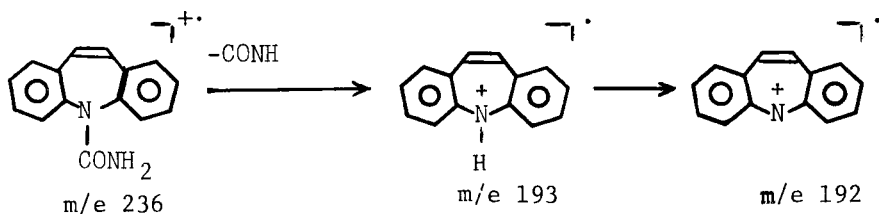


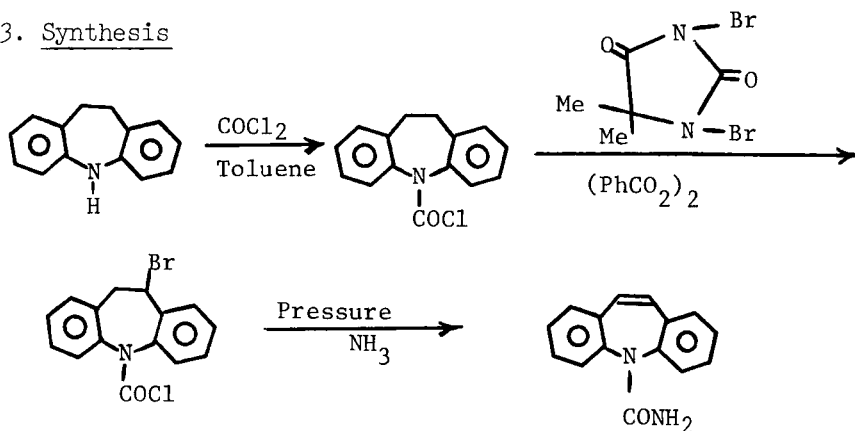
Fig. 3 - NMR spectrum of carbamazepine in CDCl<sub>3</sub> containing TMS as internal standard.

electron impact ionization shows a molecular ion  $M^+$  at  $m/e$  236 (relative intensity 9.1%) Fig. 4, and a base peak at  $m/e$  193.

Frigerio et. al (7,8) had published the mass spectrometric properties of carbamazepine and its metabolites, carbamazepine - 10,11 epoxide and 10,11-dihydro-10,11-dihydroxy-5H-dibenz [b,f] azepine-5-carboxamide. The fragmentation pattern are shown in Scheme 1. Frigerio et.al., discussed the fragmentation pattern of carbamazepine and its epoxide in details (7,8).



### 3. Synthesis



a) Carbamazepine can be synthesized as follows:-

Iminodibenzyl in toluene was treated with  $\text{COCl}_2$  to give 95% of 5-chlorocarbonyl iminodibenzyl which in turn was dissolved in  $\text{CCl}_4$  and was treated with 1,3-dibromo-5,5-dimethyl hydantoin and  $(\text{PhCO}_2)_2$  to give 90% 5-chloro-carbonyl-10-bromoimino-dibenzyl.<sup>2</sup> The latter was dissolved in xylene and heated at about  $100^\circ$  in an autoclave with gaseous  $\text{NH}_3$  to give 85% of 5-carbamoyl-5H-dibenzo [b,f] azepine (9).

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CARBAMAZEPINE

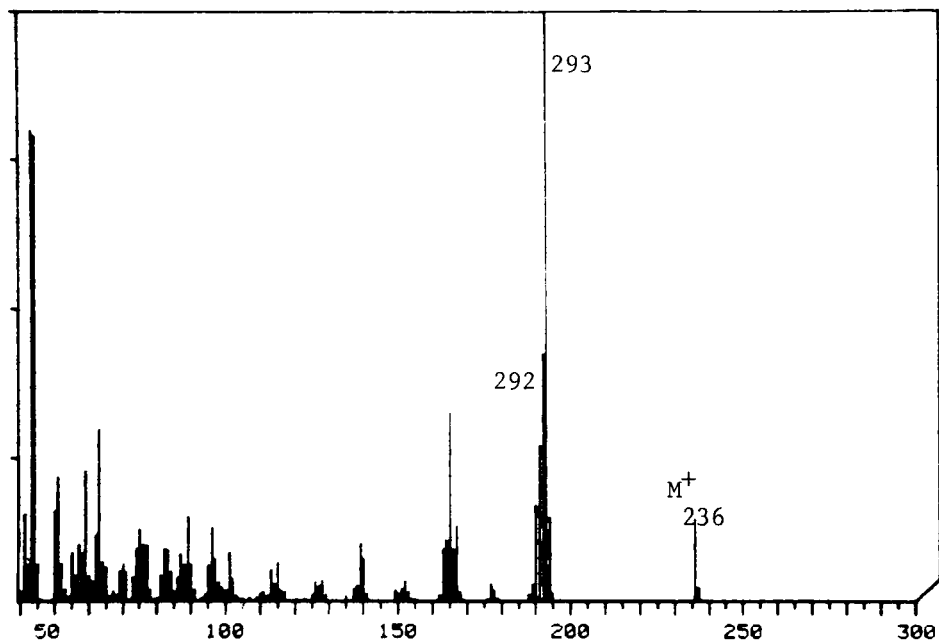
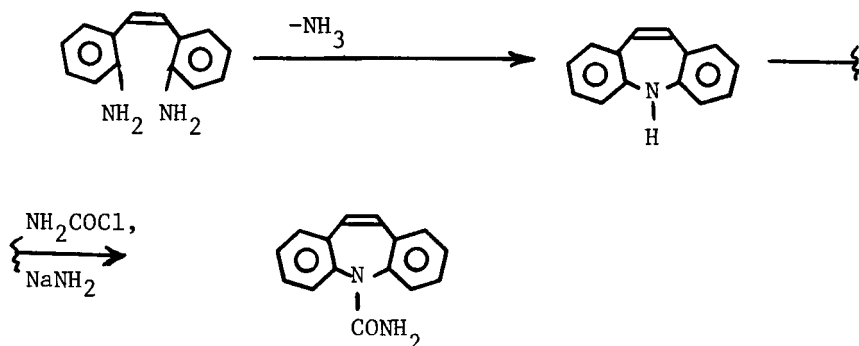


Fig. 4 - Mass spectrum of carbamazepine (EI) determined by direct probe insertion.





- b) 5H-Dibenzo [b,f] azepine, which may be prepared by thermal decomposition of 2-(0-aminostyryl)-aniline hydrochloride, is condensed with carbamoyl chloride by refluxing in an inert solvent in the presence of sodamide (10).

#### 4. Stability, Decomposition products

Carbamazepine is relatively stable drug at room temperature. However, it is recommended that it should be kept and stored in a well closed container, protected from light and in dry place.

BP 1973 (6) had described a test for identification of foreign substances namely iminodibenzyl using tlc for this purpose.

#### 5. Metabolism, Pharmacokinetics and absorption

Meinardi (11) had published a review on carbamazepine in 1972 in which he discussed the determination, metabolism and pharmacology of the drug.

Carbamazepine is readily absorbed from the gastrointestinal tract. Peak concentration in serum have been reported at about  $2\frac{1}{2}$  h after a dose. It is believed to have a half-life between 14-29 h. (12).

Studies on the plasma kinetics of carbamazepine suggested that it induced its own metabolism (13).

Frigerio et. al., (7) had isolated carbamazepine-10-11-epoxide as a urinary metabolite from humans following oral

administration. The epoxide formation was confirmed by the in vitro studies of the activity of the liver microsomal monooxygenases. SKF 525A inhibited the formation of carbamazepine oxide by 80% (14). Goenechea and Hecke-Seibicke (15) had detected seven metabolites in human urine in addition to unchanged drug by tlc. 10,11-Dihydro-10-11-dihydroxy-5H-dibenzo [b,f] azepine-5-carboxamide was identified on the basis of UV, IR, mass and NMR Spectra. Iminostilbene was also isolated as a minor urinary metabolite from rats (16).

The N-glucuronide of carbamazepine was identified in the bile of isolated perfused rat liver by the mean of permethylation GC/MS (17).

The pharmacokinetic of carbamazepine was studied in several species :-

A) Humans

Gerardin et. al., (18) had discussed the pharmacokinetics of the drug in normal humans after single and repeated doses. It was reported that the plasma concentration of the drug following single dose (100, 200, 600 mg) to normal healthy humans were fitted by a one-compartment open model. The elimination half-life after a single dose was 37.7h; it decreased during chronic treatment to a calculated value around 21h. The steady-state plasma concentration, lowers than expected from the single dose study, was adequately predicted from the single-dose data when a correction was made for the increased elimination rate constant. These findings contrast with the apparently unpredictable plasma levels reported during carbamazepine therapy.

Palmer et. al., (19) reported that following oral administration of the drug (200 mg) to two healthy fasting subjects, peak plasma concentration occurred after 6-8 h. and remained constant for 24 h before declining over the subsequent 6 days. The plasma half-life was about 36 h.

B) Rhesus monkey

The pharmacokinetics of carbamazepine (20) after a 20 mg/kg dose was administered by I.V. (5. min) infusion and orally. All semilogarithmic plasma concen-



tration-time curve after I.V. administration exhibited an irregular decay behavior in the first 3-hr period, followed by a linear disappearance phase ( $T_{1/2} = 1.0-2.4$  hr). Urinary extraction measurements confirmed the short elimination half-time and showed that  $< 1\%$  of the dose was excreted unchanged. Oral studies also yielded a short elimination half-life (1.0-1.60 hr), which was confirmed by urinary excretion measurements. The fraction of the oral dose reaching the systemic circulation ranged between 58 and 87%. Measurable (but insignificant) amounts of drug were found in the feces after I.V. and oral administrations.

C) Adult male, female and pregnant rats

After treatment with single and repeated doses of carbamazepine, male rats eliminated the drug faster than females; the total body clearance (TBC) was 16 ml/min/kg and 9.4 ml/min/kg. respectively. Two dose levels (25 and 50 mg/kg) had the same pharmacokinetic properties in young rats. Pregnant rats cleared the drug to a lesser extent than controls. Carbamazepine accelerated its own elimination after repeated administration in both adult and young rats as revealed by the shortening of its half-life and an increase of 50% in clearance. Moreover the protection against electrosock was significantly reduced after repeated administration, compared with a single-dose administration, (21).

The mean amount of carbamazepine not bound in vitro to plasma protein from 24 healthy subjects was 18.2%; the mean amount not bound in plasma from 54 patients taking the drug was 26.9% (range 7.9 to 60%). There was no significant difference in binding capacity between plasma from patients with renal disease and that from healthy subjects but the plasma from patients with level disease bound a slightly lower percentage of carbamazepine than did normal plasma (22).

6. Methods of Analysis

6.1 Spectrophotometric methods

6.11 Ultraviolet spectrophotometric methods

- a) Both BP 1973 and USP XIX (4) describe an analytical procedure for carbamazepine and its tablet formulation depending on measuring

the absorbance of the solution prepared at 285 nm. The solvent used in BP 1973 is alcohol 95% while USP XIX uses dehydrated alcohol-methanol (95:5) as a solvent system in the ultraviolet determination of carbamazepine.

- b) Fellenberg *et.al*, (23,24) reported a method for the determination of carbamazepine in blood. The method has a detection threshold of <0.1 mg/100 ml of blood. The method is based on the catalytic rearrangement of carbamazepine to 9-methylacridine. After extraction of the drug from blood with methylene chloride, which is then washed with alkali and acid. An aliquot of the extract is evaporated to dryness and the residue heated briefly with HCl at 150°. Following removal of nonspecific interference with n-heptane, the absorbance of 9-methylacridine is determined at 258 nm. The method is reported to be rapid, sensitive and specific and is suitable for routine clinical use.

#### 6.12 Nuclear magnetic resonance spectrometry

Carbamazepine has been assayed (25) in its tablet form by the application of PMR spectrometry. The method involves comparing the integral of the aromatic and olifinic protons of the drug, in the range of 6.60-7.60 ppm, to that of the singlet of known amount of hexamethylcyclotrisilazine (at 0.00 ppm) used as internal standard. The method is simple, rapid and accurate. The average percent recovery of carbamazepine from its tablets is  $96.88 \pm 0.93$ .

### 6.2 Chromatographic methods

#### 6.21 High pressure liquid chromatography

Several methods have been published for the quantitative determination of carbamazepine and its epoxide metabolite in biological fluids (plasma, serum, urine) by HPLC. Kitazawa and Komuro (26) had described a method for determination of carbamazepine and other anti-convulsant drugs in human blood plasma. The method involved two step extraction procedures with chloroform and use of 2x50 cm long stainless steel column packed with an

anion exchange resin. The mobile phase was 4 mM ammonium phosphate buffer solution of pH 6.2 at a flow rate of 0.40 ml/min.

The results presented showed linear calibration curves and quantitative determination as low as 1.0 µg/0.5 ml plasma. The method was efficient to detect the drug in plasma after therapeutic clinical doses.

Eichelbaum and Bertilsson (27) described a method using HPLC-mass spectrometry for simultaneous determination of carbamazepine and its active 10, 11-epoxide metabolite in plasma. The method required no derivatization and had a lower limit of sensitivity of 4 ng for carbamazepine and 4 ng for its metabolite. The method is very specific and had a precisions of 2.2% for the drug and 4.2% for its metabolites.

Karba Marton (28) published a method for determination of carbamazepine in the whole blood by HPLC. The drug was well separated from normal blood constituents in less than 8 minutes. The sensitivity of this method is 0.25 mg of the drug/l in a 2 ml sample, and the lower limit of detection is 100 ng.

Another method (29) was reported for determination of carbamazepine in plasma is described in which the drug was extracted with ether, isolated with Lichrosorb RP8 and detected by UV spectroscopy at 280 nm. The recovery of the drug was at concentration 1-2 µg/ml. There was no interference by the drug metabolite or endogenous plasma components.

#### 6.22 Paper chromatography

Clarke (5) described several solvent systems used for paper chromatographic detection of carbamazepine as shown in Table 1.

#### 6.23 Thin Layer Chromatography

Carbamazepine was determined among other anticonvulsant agents in serum by tlc. The serum was extracted with toluene and the dried extract was dissolved in chloroform and spotted on to a tlc

Table 1

Solvent System	Visualizing agent	R <sub>f</sub>
Citric acid : H <sub>2</sub> O : n-butanol (4.8 gm : 130 ml : 870 ml)	Ultraviolet blue fluorescence	0.34
Acetate Buffer (pH 4.58)	Ultraviolet, blue fluorescence	0.29
Phosphate Buffer (pH 7.4)	Ultraviolet, blue fluorescence.	0.30

plate. After development, the plate was scanned at 215 nm without staining. Most of the interfering substances that occur naturally in serum were soluble in and eliminated by the liq. front. (30).

Clarke (5) reported the use of a solvent system consisting of strong ammonia solution : methanol (1.5 : 100), the solvent system is recommended to be changed after two runs. Carbamazepine gives an R<sub>f</sub> value of 0.73. The chromatogram (Silicagel G) is visualized by potassium permanganate spray.

#### 6.24 Gas-Liquid Chromatography

Gas-Liquid chromatography is considered to be the main procedure for the quantitation and analysis of carbamazepine specially in biological fluids and tissues.

The drug has been determined by several authors (31, 32, 33, 34) through derivatization to its methylated derivative. The methods reported show lower limit of detection of about 0.5 mg/litre. Carbamazepine had been determined by GLC without derivatization on several stationary phases as shown in Table 2.

Toseland et al., (38) described the determination of carbamazepine among other anticonvulsants and barbiturates in plasma and tissue using the nitrogen flame detector.

Table 2

Stationary phase	Detector used	Reference
3% OV - 17	Flame ionization.	(35)
2% SP - 1000	Isothermal	(36)
Cab-0-Sil deactivated with benzyltriphenyl phosphonium chloride and OV - 225	Isothermal	(37)

Marozzi *et. al.*, (39) had reported the gas chromatographic retention indexes ( $I_R$ ) of 232 compounds of toxicological interest which were determined isothermally at 180° on SE 30, OV.1, OV-17, among which was carbamazepine.

Clarke (5) reported the retention time of carbamazepine to be 0.81 relative to codeine and 3.76 relative to diphenhydramine using 2.5% SE-30 on 80-100 chromosorb W AWHMDS and the conditions specified in the monograph.

#### ACKNOWLEDGEMENTS

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# CEFACLOR

*Leslie J. Lorenz*

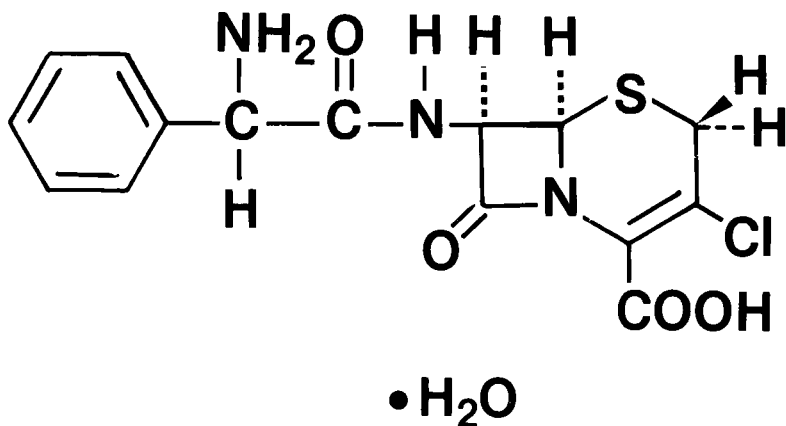
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## 1. Description

## 1.1. Name

Cefaclor is 3-chloro-7-d-(2-phenylglycinamido)-3-cephem-4-carboxylic acid, monohydrate.

## 1.2. Structure, formula and molecular weight



molecular weight 385.82

## 1.3. Appearance

Cefaclor is a white to cream colored crystalline powder. The material is odorless going to slightly sulphurous.

## 2. Physical properties

## 2.1. Infrared spectrum

The infrared spectrum of cefaclor monohydrate in a potassium bromide pellet is presented in figure 1. An interpretation of the spectrum is given in table 1.

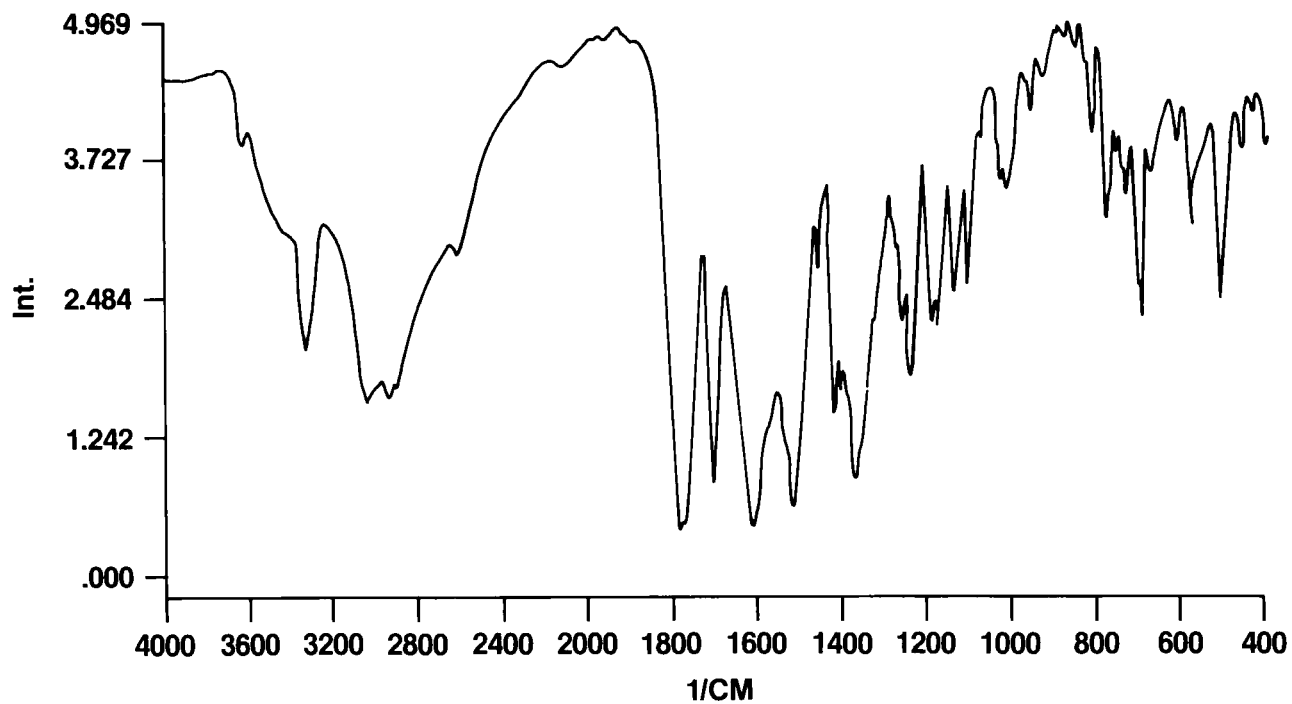


Figure 1 The Infrared Spectrum of Cefaclor in a KBr Pellet

Table 1

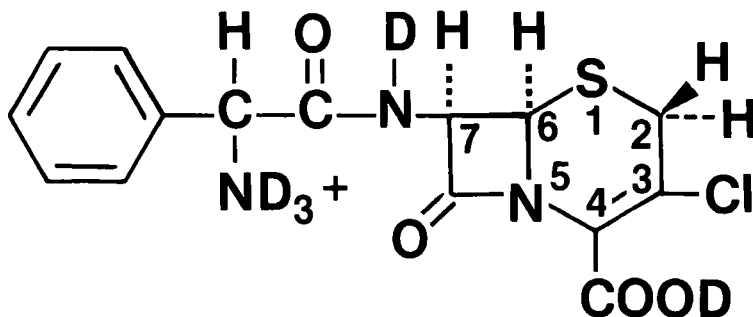
Wavelength ( $\text{cm}^{-1}$ )	Assignment
3680-3000 (series of broad bands)	OH from $\text{H}_2\text{O}$ and amide NH stretch
2580 (broad)	$\text{CO}_2^-$
1775 (strong)	$\beta$ -lactam C=O stretch
1693 (strong)	amide C=O stretch
1600 (strong)	$\text{C}=\text{O}$ carboxylate stretching
1560 (weak)	aromatic C=C
1500 (medium)	aromatic C=C
1365 (strong)	$\text{CO}_2^-$ (sym)
697 (sharp)	C-Cl

## 2.2. Nuclear magnetic resonance spectrum

Figure 2 shows the proton magnetic resonance spectrum of cefaclor. The spectrum was recorded on a 60 MHz instrument. An interpretation of the spectrum is presented in table 2.

Table 2

### Proton Magnetic Resonance Spectrum



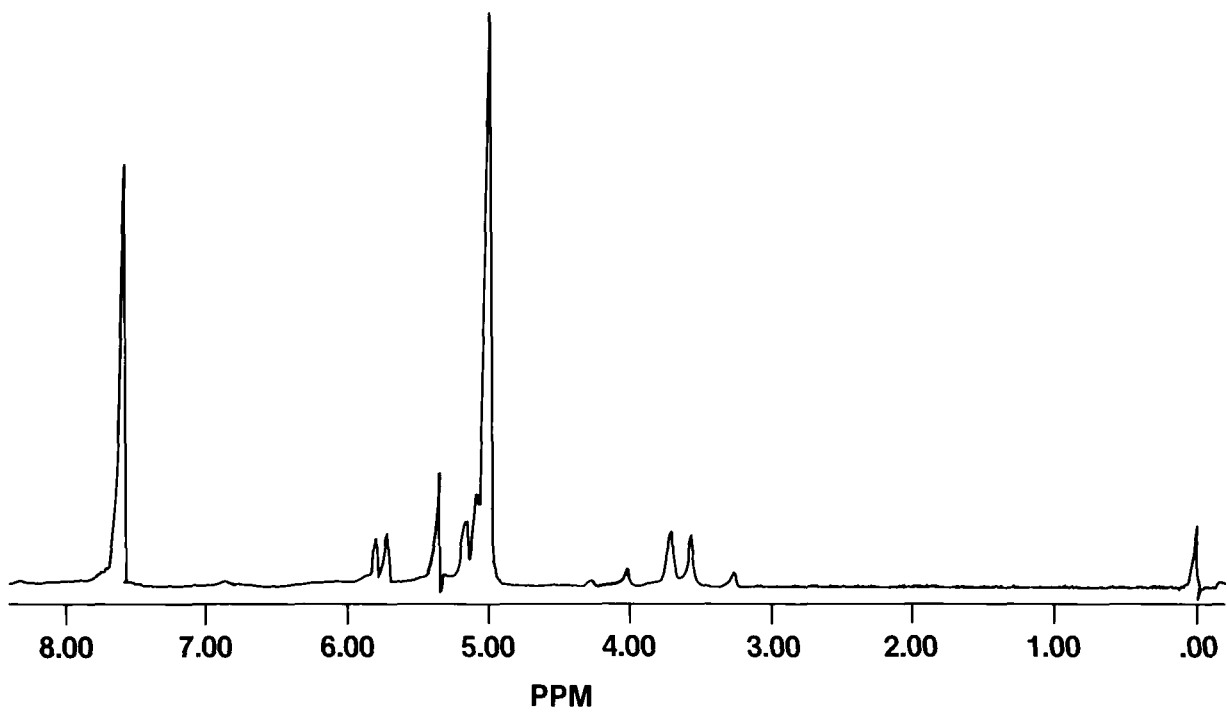


Figure 2 The NMR Spectrum of Cefaclor in D<sub>2</sub>O+DCl



## Peak Assignments

<u>ppm</u>	<u>Multiplicity</u>	<u>Assignment</u>
3.66	quartet AB J=19Hz	CH <sub>2</sub> (2)
5.17	doublet	H (6)
5.35	singlet	Ø-CH-CO-   ND <sub>3</sub>
5.78	doublet J=5HZ	H (7)
7.60	singlet	phenyl

## 2.3. Mass spectrum

Cefaclor is amenable to field desorption techniques for obtaining a meaningful mass spectrum. Using this technique, a small quasi-molecular ion is seen for cefaclor at m/e of 369 which corresponds to the molecular weight of cefaclor anhydrate. The major ion in the mass spectrum appears at m/e of 331. This is caused by the loss of HCl from the molecule. The only other significant ion in the spectrum appears at m/e of 287. This ion corresponds to a molecule which has lost HCl and decarboxylated.

## 2.4. Ultraviolet spectrum

Figure 3 shows the ultraviolet spectrum for cefaclor. The chromophores in cefaclor are 3-cephem, phenyl and amide. Of these, only the 3-cephem group contributes significantly about 220 nm. The  $\pi \rightarrow \pi^*$  3-cephem transition has  $\epsilon(265 \text{ nm}) \approx 8400$ . There is a  $n \rightarrow \pi^*$  transition at about 230 nm due to the 3-cephem group. The phenyl group has a weak absorption at 260 nm with  $\epsilon \approx 200$ .

## 2.5. Optical rotation

The specific rotation for cefaclor determined on a one percent solution of cefaclor in 0.1 M hydrochloric acid at Na<sup>20</sup><sub>D</sub> is +105.6° on an anhydrous basis.

## 2.6. Differential thermal analysis

The thermogram of cefaclor generally shows a small broad endotherm between 40°C and 120°C corresponding to the loss of water and other volatiles from the sample. The major endotherm in the DTA curve for cefaclor is observed around 220°C where the material decomposes.

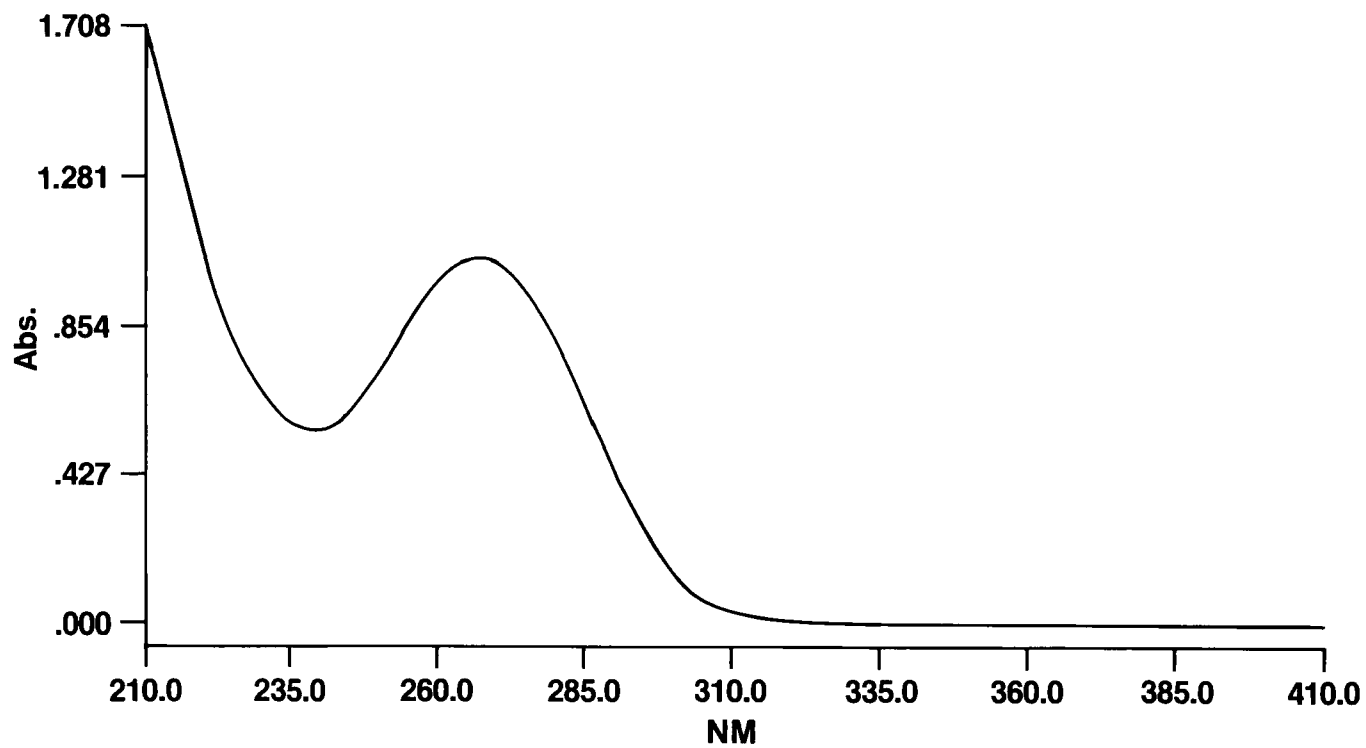


Figure 3 The Ultraviolet Spectrum of Cefaclor in 0.1M HCl

### 2.7. Thermogravimetric analysis

Cefaclor gives a reasonable thermogravimetric curve and shows a loss of water and other volatiles from about 40°C to 120°C. At about 180°C cefaclor samples begin to lose weight indicating the beginning of decomposition of the sample.

### 2.8. Dissociation constant pKa

The following dissociation constants have been determined for cefaclor:

<u>Solvent</u>	<u>pKa</u>	
	<u>Carboxyl</u>	<u>Amino</u>
H <sub>2</sub> O	1.5±0.2	7.17
66% DMF	4.33	7.34

### 2.9. Solubility properties

The solubility properties of cefaclor are described in table 3.

Table 3

<u>Solvent</u>	<u>Solubility mg/ml</u>
Water	10.0
pH 1.2 (USP XIX)	>5 but <10
pH 4.5 (USP XIX)	4
pH 7.0 (USP XIX)	>5 but <10
Methanol	<0.5
Octanol	<0.5
Isopropanol	<0.5
Diethyl ether	<0.5
Ethyl acetate	<0.5
Chloroform	<0.5
Benzene	<0.5
Cyclohexane	<0.5

### 2.10. Crystal properties

Polymorphs of cefaclor are possible. Such polymorphs are a function of the solvent from which cefaclor is crystallized. The only polymorph of general importance is cefaclor monohydrate. The X-ray powder diffraction data for cefaclor monohydrate are given in table 4.

Table 4

## X-ray Powder Diffraction Data

Cefaclor Monohydrate

 $\lambda=1.5418$ 

<u>"d" Value (Å)</u>	<u>Intensities (I/I<sub>0</sub>)</u>
12.90	0.75
10.05	0.17
6.58	0.13
6.08	0.13
5.42	0.96
5.01	1.00
4.75	0.04
4.06	0.54
3.86	0.04
3.69	0.29
3.53	0.58
3.41	0.04
3.29	0.17
3.23	0.13
3.13	0.04
2.99	0.21
2.81	0.25
2.67	0.08
2.52	0.08
2.48	0.04
2.35	0.17
2.26	0.17
2.15	0.04
2.07	0.08
1.99	0.21
1.94	0.08

## 3. Chemical synthesis

Figure 4 provides a flow sheet of the chemical synthesis for cefaclor. In this procedure, penicillin V (1) is esterified with p-nitrobenzyl bromide (PNB-Br) and oxidized with peracetic acid to give penicillin sulfoxide ester (2). Ring expansion and ozonolysis provide the 3-hydroxy-3-cephem sulfoxide (4). Sulfoxide reduction and enol chlorination occurs with phosphorous trichloride in N,N-dimethylformamide. Side chain cleavage is accomplished with phosphorous pentachloride and pyridine followed by alcoholysis with isobutyl alcohol. The resulting nucleus hydrochloride (5) is neutralized with

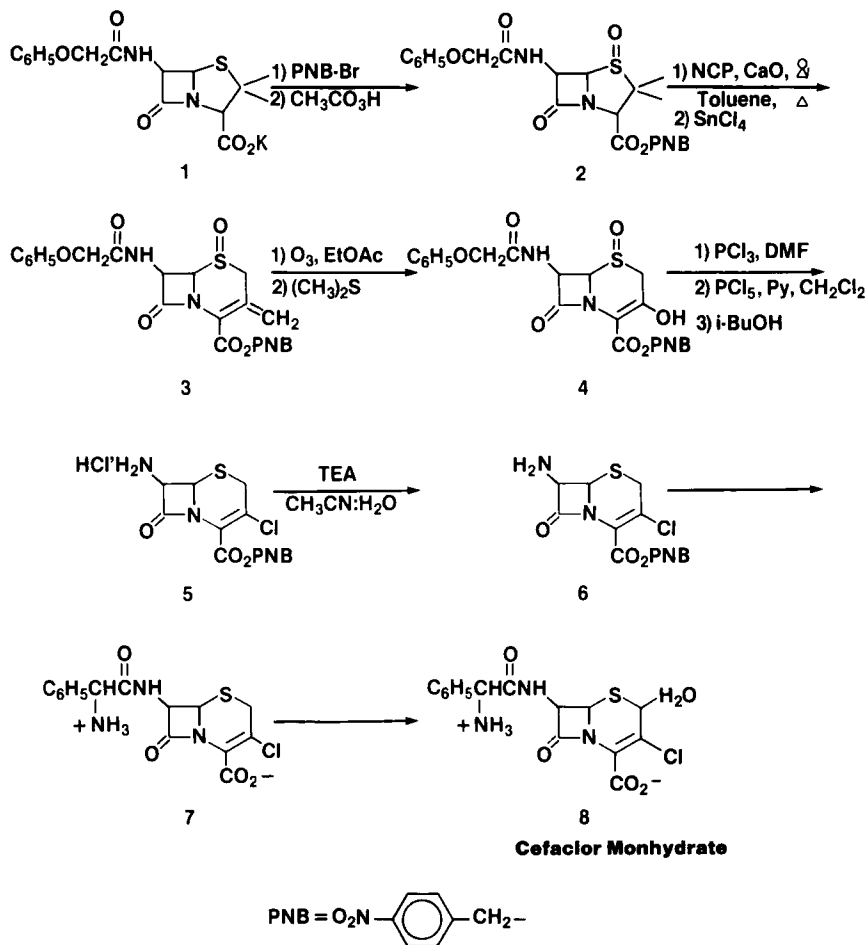


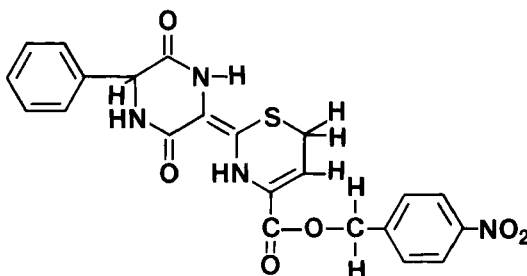
Figure 4 The Chemical Synthesis of Cefaclor

triethylamine and acylated with a N-protected D-phenylglycine to give cefaclor (7). This is then hydrated in water to yield the desired monohydrate (8).

#### 4.1. Bulk stability

Cefaclor is a reasonably stable molecule in the dry state. When cefaclor is present in the monohydrate crystalline form in the dry powder, two year stability can be easily obtained. The powder becomes lightly yellow upon aging, however, little decrease in the potency of cefaclor is observed.

On degradation, cefaclor appears to lose HCl quite easily. Further degradation steps seem to be quite rapid and no other compounds have been isolated. In an attempt to generate such compounds, some studies have been carried out on the p-nitrobenzylester of cefaclor. This study showed that cefaclor can undergo intramolecular nucleophilic attack by the side chain amine group to produce a diketopiperazine with the following structure (1):



#### 4.2. Solution stability

Cefaclor is stable in solutions of pH not higher than 4.5. Solutions prepared in pH 2.5 and 4.5 buffers contain at least 90 percent of their initial activity after 72 hours at 4°C (2). In neutral or alkaline solutions, cefaclor undergoes a rapid loss of activity. When held in Mueller-Hinton broth at 37°C overnight, 30 to 60 percent of the initial activity of the solution is lost (3,4).

### 5. Drug metabolism

When cefaclor was administered to normal volunteers, peak serum concentrations of cefaclor occurred about one hour after administration. A 250 mg dose gave an approximate peak level of 7 mcg/ml. A 500 mg dose gave an approximate peak level of 13 mcg/ml, and a 1 gram dose gave an approximate peak level of 23 mcg/ml (5,6). The mean serum

half life of cefaclor in normal adult volunteers as determined by several investigators ranges from 29 to 60 minutes (5-10).

Cefaclor is rapidly excreted in the urine. In several studies, 38 to 54 percent of the drug was detected in the urine in the first two hours after administration (7). After eight hours 43 to 79 percent of the drug was found in the urine (6,10).

These studies would tend to indicate that about 85 percent of the drug is excreted into the urine as the unchanged drug. When cefaclor is metabolized in the body or natural degradation occurs, the effect on the molecule is severe and no recognizable products have been found.

## 6. Methods of analysis

### 6.1. Identification tests

#### 6.1.1. Infrared

The infrared spectrum of a sample in a potassium bromide pellet may be used for identity. In such cases, the infrared spectrum compares favorably with the cefaclor reference spectrum over the range of 2.5 to 16 microns when recorded in a similar manner.

#### 6.1.2. Nuclear magnetic resonance

The NMR spectrum of a cefaclor sample dissolved in deuterium oxide with deuterium chloride present, over the range of 0 to 10 ppm has chemical shifts and integrations which compare favorably to a cefaclor reference material handled in like manner.

### 6.2 Quantitative tests

#### 6.2.1. Microbiological

For bulk and formulated products, either an agar diffusion assay with Bacillus subtilis (ATCC 6633) (2) or an automated turbidimetric assay (AUTOTURB®) with Staphylococcus aureus (ATCC 9144) (2) may be used for assay of cefaclor. Agar diffusion assays with either B. subtilis (2,8,10,11,12,13) or Sarcina lutea (ATCC 9341) (2,13) are used to assay the antibiotic in tissues and biological fluids. The greatest sensitivity is obtained with S. lutea as concentrations of cefaclor as low as 0.025 mcg per mg may be determined (2).

### 6.2.2. High performance liquid chromatography

HPLC is the technique of choice for determining the purity of cefaclor in raw material, formulated products and in body fluids. Cefaclor is run in an acidic medium on a reverse phase column. A Waters Microbondapak® C18 or other alternative column with similar retention characteristics is used to determine the purity. The approximate solvent system consists of 2 parts glacial acetic acid, 12 parts acetonitrile and 86 parts water. The substance is usually monitored at 254 nm; however, slightly improved sensitivity can be obtained at about 265 nm. The samples are dissolved in an acidic media such as a pH 4.6 buffer or in dilute formic or acetic acid.

### 6.2.3. Iodometric titration

An iodometric titration procedure similar to that used for cephalixin (14) has been adapted for cefaclor. In this procedure as with other cephalosporins, the intact antibiotic does not consume iodine, while the alkali-hydrolysis product of cefaclor does. The alkaline hydrolysis of cefaclor results in the cleavage of the  $\beta$ -lactam ring. This product then reacts with iodine to give a quantitative titration procedure for cefaclor. This test can be run in a manual mode as well as in an automated mode with behavior similar to that of cephalixin (15). This test is not necessarily a stability indicating test for cefaclor since any molecule with an intact  $\beta$ -lactam moiety will give a test in this procedure.

### 6.2.4. Colorimetric determination with hydroxylamine

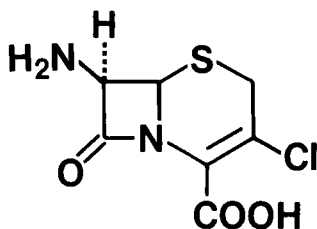
The reaction of hydroxylamine with cefaclor has been used to determine the drug (16). The method is based on the fact that hydroxylamine cleaves the  $\beta$ -lactam ring (pH 7.0) to form a hydroxamic acid. This hydroxamic acid forms a colored complex with ferric ion. Again any entity having the  $\beta$ -lactam ring intact will give a test with this procedure. Thus, this test may not be a stability indicating test for cefaclor.

## 6.3. Impurities

### 6.3.1. Colorimetric determination of 3-chloro nucleus



The 3-chloro nucleus of cefaclor



has a free  $\alpha$ -amino group adjacent to a  $\beta$ -lactam. This type of moiety is sensitive to a test with ninhydrin to form a colored reaction product. The reaction is carried out in a citrate buffer and a pH of about 3.0. After 30 minutes for color development, the absorbance is read at 560 nm.

#### 6.3.2. Phenylglycine

Phenylglycine content of cefaclor can be determined by a high performance liquid chromatographic procedure. In this procedure, phenylglycine is determined using a Waters Microbondapak® C18 column or other similarly suitable reverse phase column. The eluting solvent consists of 0.01 M potassium dihydrogen phosphate titrated to a pH of 2.7 with phosphoric acid and 1 percent by volume of acetonitrile. The column eluent is monitored at 220 nm for determination of phenylglycine. Phenylglycine is thus determined versus the response of a phenylglycine standard handled in like manner.

As might be expected, cefaclor elutes very late in such a system and may be removed from the column in a quick gradient or step gradient procedure where the acetonitrile composition of the mobile solvent is increased until the elution of the cefaclor has been completed.

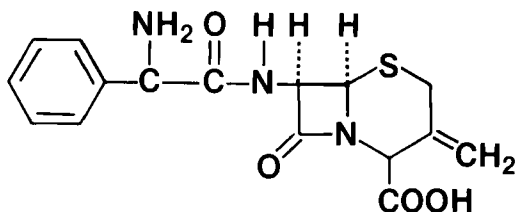
#### 6.3.3. Cephalixin

Cephalixin might be a potential impurity arising from the rearrangement of the 3-exomethylene intermediate in the synthesis of cefaclor. Trace levels of cephalixin may be determined by high performance liquid chromatograph techniques. To determine the cephalixin content of cefaclor, a reverse phase system is employed using a Waters Microbondapak® C18 or other suitably similar HPLC column. The eluting solvent consists of 91 parts water, 4 parts acetonitrile and 5 parts glacial acetic acid. The column eluent is monitored at 265 nm for determination of cephalixin. The response obtained at the

elution volume of cephalixin is compared to the response of a sample containing cephalixin at a known composition to determine the content of cephalixin in the cefaclor sample.

#### 6.3.4. 3-Exomethylene analog of cefaclor

The 3-exomethylene analog of cefaclor



is a potential impurity which may carry through the synthesis if the ozonolysis of the corresponding intermediate should be incomplete. This compound is determined by high performance liquid chromatography. To determine this compound, a Waters Microbondapak® C18 or other suitably similar reverse phase column is employed. The eluting solvent for this determination is 1 part glacial acetic acid, 7.5 parts methanol, and 91.5 parts water. The column eluent is monitored at 225 nm. The 3-exomethylene analog is determined by measuring the response of the 3-exomethylene peak in the sample chromatogram and comparing it to the response of a sample with a known content of the 3-exomethylene analog.

#### 6.3.5. Other impurities

Gradient HPLC procedures can be utilized for the determination of other unidentified impurities in cefaclor. In this procedure, a Waters Microbondapak® C18 column, or a Dupont Zorbax® TMS column or other suitably similar HPLC reverse phase column is employed. A linear gradient is run from 2 percent glacial acetic acid in water to 2 percent glacial acetic acid in acetonitrile. Most compounds of interest elute early in the system so a 2 percent change per minute is used for 25 minutes followed by a faster slope such as 5 percent change per minute for the remainder of the chromatogram. Samples are prepared at about 25 mg per ml in formic acid and about 20 ul of such a solution is chromatographically examined. The column eluent is monitored at 254 nm. The peak areas of all unidentified peaks are integrated and compared to the response of a cefaclor standard at about one percent of the concentrated solution. The assumption is made that

all other impurities have similar spectral properties as cefaclor and an approximation of their levels in the sample can then be made.

#### 7. Determination in body fluids

Microbiological and high performance liquid chromatographic procedures have been employed for the determination of cefaclor in biological fluids. Generally, protein has been precipitated from the samples by classical means and the fluids are then examined by one of these techniques. When handling biological fluids which contain cefaclor, care must be taken so that the solutions are kept cold in an ice bath or frozen from the time of sampling to the time of assay. Also, if possible, it is advisable to acidify the samples to prevent loss of cefaclor due to its instability at higher pH's.

#### 8. Acknowledgements

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- L. G. Tensmeyer for the infrared assignments.
- T. C. Troxell for the ultraviolet spectral interpretation.
- P. G. Wassel for the cephalixin test and the 3-chloronucleus test.
- C. L. Winely for the microbiological assay portions.

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# CEFAMANDOLE NAFATE

*Rafik H. Bishara and Eugene C. Rickard*

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## Introduction

Cefamandole nafate is a semisynthetic broad-spectrum cephalosporin antibiotic for parenteral administration. The dosage form of cefamandole nafate also contains 63 mg of sodium carbonate per gram of cefamandole free acid activity (0.275 moles of sodium carbonate per mole of cefamandole free acid activity). After addition of diluent, cefamandole nafate rapidly hydrolyzes to cefamandole, and both compounds have microbiologic activity in vivo.

## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Name

7-D-Mandelamido-3- <<(1-methyl-1H-tetrazol-5-yl)thio>methyl>-3-cephem-4-carboxylic acid, formate (ester), sodium salt

7-<D- <(Formyloxy)phenylacetyl>amino>-3- <<(1-methyl-1H-tetrazol-5-yl)thio>methyl>-3-cephem-4-carboxylic acid, sodium salt

7-D-Mandelamido-3-[[ (1-methyl-1H-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylate formate(ester)

#### 1.1.2 Nonproprietary Name

Cefamandole nafate

#### 1.1.3 Proprietary Name

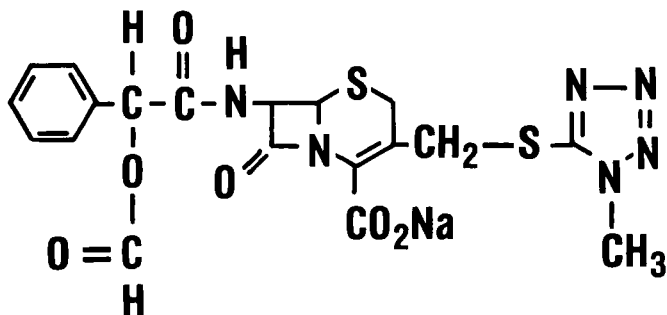
Mandol <sup>®</sup>, Mandokef <sup>®</sup>

1.2 Formula

1.2.1 Empirical

$C_{19}H_{17}N_6O_6S_2 \cdot Na$  Salt

1.2.2 Structural



1.3 Molecular Weight

512.49

1.4 Appearance, Color, Odor, and Taste

White to off-white, odorless powder with a slightly bitter taste.

2. Physical Properties

2.1 Melting Range

Cefamandole nafate starts to discolor with evolution of gas at about 190°C under USP conditions for Class I substances (1).

2.2 Simple Solubility Profile

The sample is sonicated for one minute at ambient temperature.

<u>Solvent</u>	<u>mg/ml</u>
Water	≥333-<1000
pH 1.2 (USP XIX)	<0.5
pH 4.5 (USP XIX)	≥333-<1000
pH 7.0 (USP XIX)	≥333-<1000
Methanol	≥10-<33.3
Octanol	<0.5
Isopropanol	<0.5
Diethylether	<0.5
Ethylacetate	<0.5
Chloroform	<0.5
Benzene	<0.5
Cyclohexane	<0.5

### 2.3 Specific Rotation

Rotation measured at sodium D line (approximately 589nm) of a 10% solution of cefamandole nafate in pH 5.0 acetate buffer (1.04M) is  $-38 \pm 5$  calculated on an anhydrous basis. It is to be noted that the specific rotation is a function of concentration. In unbuffered solutions or solutions buffered at pH 6.0-7.5, the conversion of cefamandole nafate to cefamandole causes a drift, with time, of the measured rotation. There is minimal drift and pH dependence in the range of pH 4.5-5.5.

### 2.4 pH Range

The pH of a 10% aqueous solution is between 3.5 and 7.0.

### 2.5 Dissociation Constant

The carboxylate  $pK_a$  of cefamandole nafate is about 2.6-2.9 as determined by aqueous titration or 3.0 as determined by spectrophotometry (2).

### 2.6 Thermal Analysis

#### 2.6.1 Differential Thermal Analysis

A DTA thermogram of cefamandole nafate, at a heating rate of  $5^\circ\text{C}$  per minute in a nitrogen atmosphere of 40cc per minute, shows (figure 1) an exotherm at  $207^\circ\text{C}$  indicating decomposition.

#### 2.6.2 Thermogravimetric Analysis

A TGA thermogram of cefamandole nafate, run simultaneously with the above DTA, shows (figure 1) a weight loss beginning at  $63^\circ\text{C}$  resulting in a 0.2% loss at  $137^\circ\text{C}$ . At  $163^\circ\text{C}$  another loss begins resulting in a continuous loss through decomposition.

### 2.7 Crystallinity

#### 2.7.1 Crystalline Habit

The anhydrate form ( $\gamma$ ) of cefamandole nafate generally crystallizes as small needles.

#### 2.7.2 X-Ray Powder Diffraction

The following data describe the pattern for the anhydrate form ( $\gamma$ ) of cefamandole nafate, where  $d$  is equal to the interplanar spacing measured in terms of Angstroms ( $\text{\AA}$ ). The ratio  $I/I_1$  is the intensity of the X-ray maxima based upon a value of 100 for the strongest line.



Cu-Ni- $\lambda$  1.5405 Å

<u>d</u>	<u>I/I<sub>1</sub></u>	<u>d</u>	<u>I/I<sub>1</sub></u>
17.80	30	3.72	100
11.76	30	3.51	5
9.39	10	3.32	2
7.49	70	3.06	10
7.18	20	2.91	15
6.20	15	2.83	15
5.52	40	2.75	10
5.00	40	2.56	5
4.74	20	2.36	10
4.54	80	2.17	10
4.20	50	2.11	10
3.98	10		

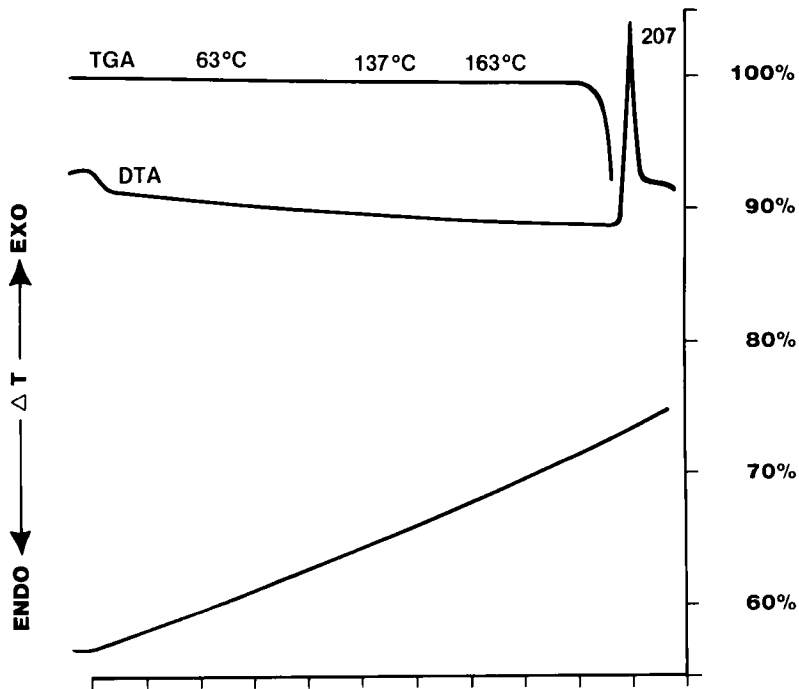


Figure 1. Thermogravimetric Analysis and Differential Thermal Analysis Thermograms of Cefamandole Nafate

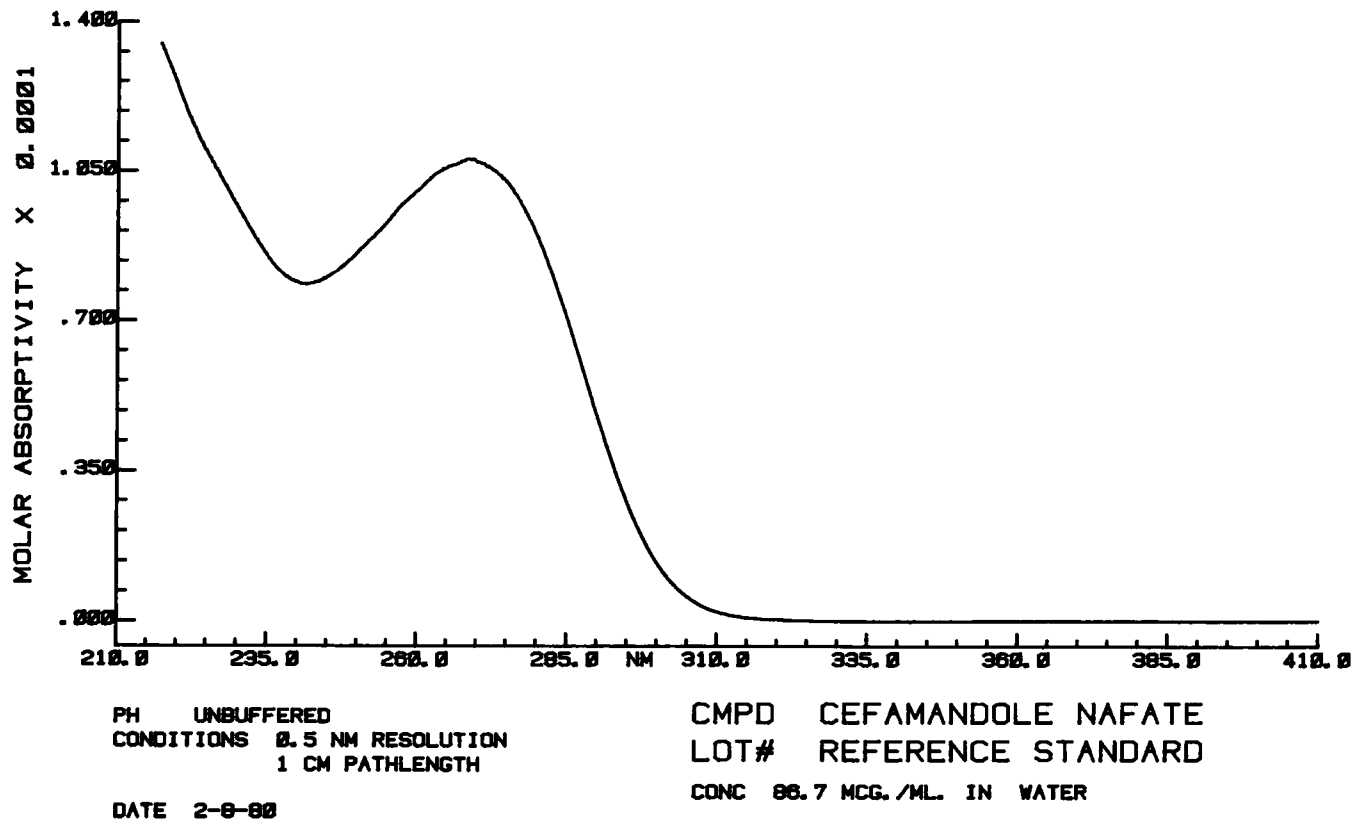


Figure 2. Ultraviolet Spectrum of Cefamandole Nafate

## 2.8 Ultraviolet Spectrum

The ultraviolet spectrum of cefamandole nafate in water is given in figure 2. The spectrum exhibits a maximum at 269nm with a molar absorptivity of 10,800 ( $E_{1\text{-cm}/1\%}$  about 211). The chromophores in cefamandole nafate are 3-cephem, thiotetrazole, phenyl, amide, and ester. Of these, only the 3-cephem and thiotetrazole make significant contributions above about 225nm. For the 3-cephem group in  $H_2O$ ,  $\epsilon$  (261 nm) = 9200 is expected from a  $\pi \rightarrow \pi^*$  transition; there is probably a  $\pi \rightarrow \pi^*$  transition at about 230 nm. also. Thiotetrazole has a 245 nm peak with  $\epsilon$  about 12,800. On substitution into the antibiotic, the peak apparently red shifts to around 275 nm with a dramatic intensity decrease to  $\epsilon$  about 4000. This can be seen by comparison of -OH versus thiotetrazole substitution.

## 2.9 Circular Dichroism Spectrum

The circular dichroism (CD) spectrum of cefamandole nafate in water is given in figure 3. The  $\Delta\epsilon$  maxima and zeros are:

$\lambda, \text{nm}$	$\Delta\epsilon$
264	7.11
246.7	0
228.5	-19.81
209	-12.70
195	0

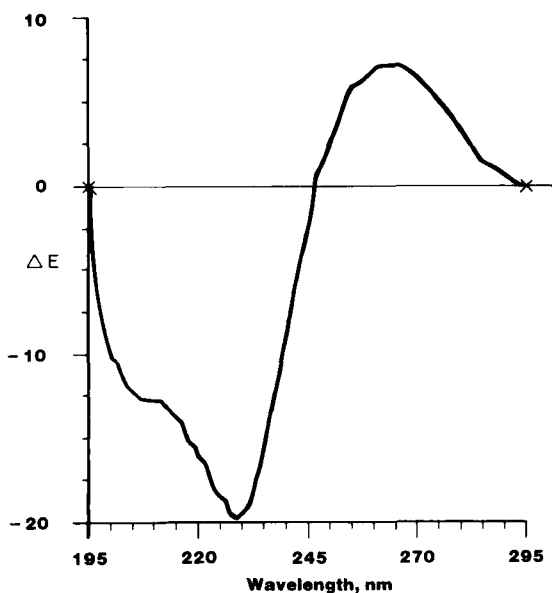


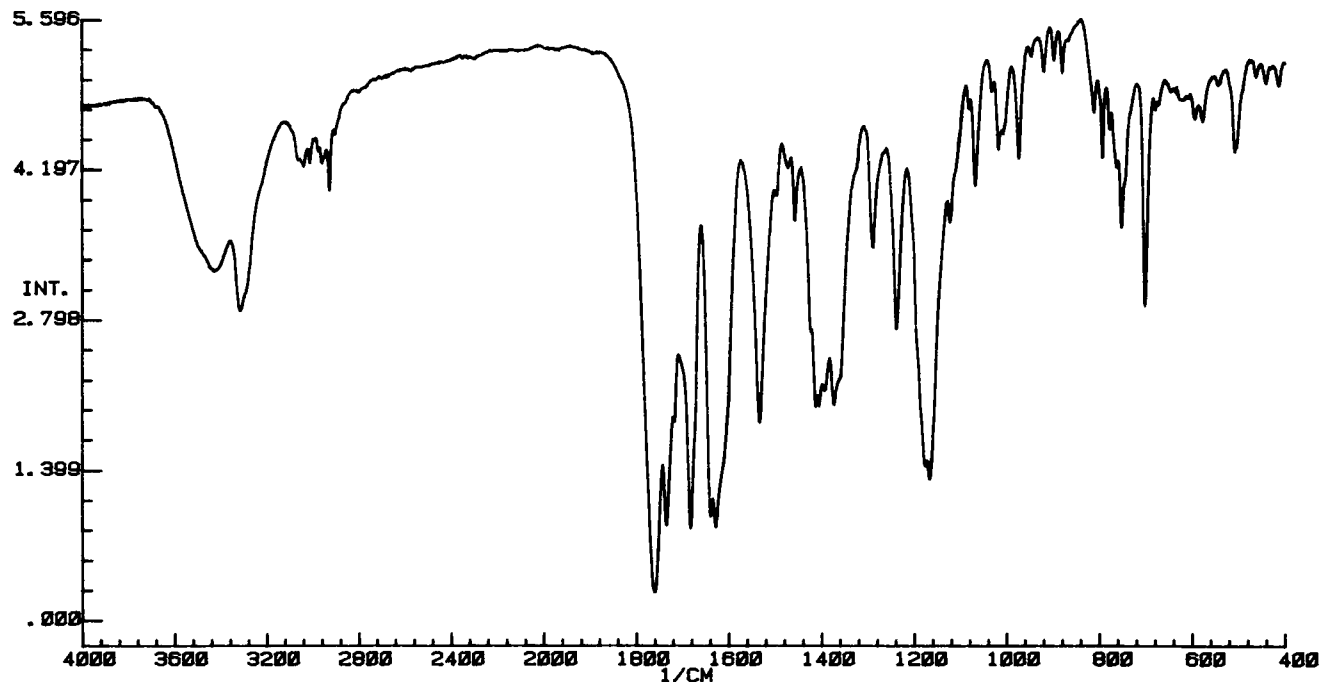
Figure 3. Circular Dichroism Spectrum of Cefamandole Nafate

The CD of cefamandole above 220 nm appears to be that typical to the 3-cephem group. The 228.5 nm negative CD comes from the  $n \rightarrow \pi^*$  3-cephem transition. The positive CD peak at 264 nm probably locates the position of the  $\pi \rightarrow \pi^*$  3-cephem transition. The fact that the absorption peak is at 269 nm rather than 264 is probably due to the thiotetrazole absorption, from which CD is either weak or absent. Similarly, the thiotetrazole group is responsible for the absorption above 295 nm, where the CD is zero. The lack of optical activity (CD) in the thiotetrazole 275 nm transition may result from its conformational mobility, as implied by molecular models (3).

### 2.10 Infrared Spectrum

The infrared spectrum of cefamandole nafate in a potassium bromide pellet is given in figure 4. Major band assignments are as follows:

<u>Infrared Absorption, <math>\text{cm}^{-1}</math></u>	<u>Group Responsible</u>
3500, very broad	H-bonded $\text{H}_2\text{O}$
3260, sharp	H-bonded <u>trans</u> N-H in secondary amide
3040-3020	CH in mono-substituted phenyl ring
2940	CH stretch in $\text{N-CH}_3$ and
2880	$\text{S-CH}_2$
1755	carbonyl in $\beta$ -lactam
1712	carbonyl in ester
1668	carbonyl in amide (termed Amide I)
1620 } 1610 } 1600 }	{ carboxylate salt C=C, conjugated to acid group C=C in phenyl ring
1530	Amide II
1490	aromatic C=C
1450	$\text{N-CH}_3$
1385 or 1357	carboxylate salt
1174	C=O in ester
1100	tertiary nitrogen, $\text{-N-}$
745	CH pattern for mono-
692	substituted phenyl ring



PATH PELLET  
ISM# 16 MINUTE SCAN TIME  
CONDITIONS 3 WAVENUMBER RESOLUTION

CMPD CEFAMANDOLE NAFATE  
LOT# REFERENCE STANDARD  
CONC 0.87 MG. IN KBr

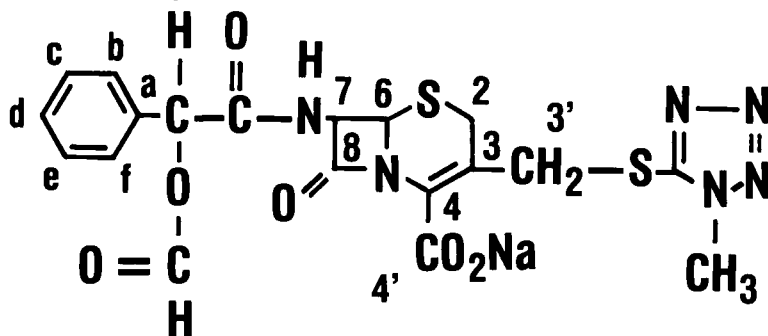
DATE 4-20-79

Figure 4. Infrared Spectrum of Cefamandole Nafate

## 2.11 Nuclear Magnetic Resonance Spectrum

### 2.11.1 Proton NMR

The 60 MHz proton NMR spectrum of cefamandole nafate in deuterated dimethylsulfoxide is given in figure 5. Assignment of the resonances are as follows:



#### Description of Resonance:<sup>a</sup>

#### Assignment:

d/9.38 p.p.m. ( $J = 8.5$ )

NH

s/8.37 p.p.m.

CHO

m/7.45 p.p.m. (5H)

aromatic protons

s/6.13 p.p.m.

-CH-CO  
|  
O

dd/5.53 p.p.m. ( $J = 8.5, 5$ )

H-7

d/4.87 p.p.m. ( $J = 5$ )

H-6

AB-system/4.32 p.p.m. (2H) ( $J_{AB} = 12$ )

CH<sub>2</sub> (3')

s/3.90 p.p.m. (3H)

NCH<sub>3</sub>

m/ca. 3.4 (2H)<sup>b</sup> ( $J_{AB} \approx 18$ )

CH<sub>2</sub> (2)

m/2.53

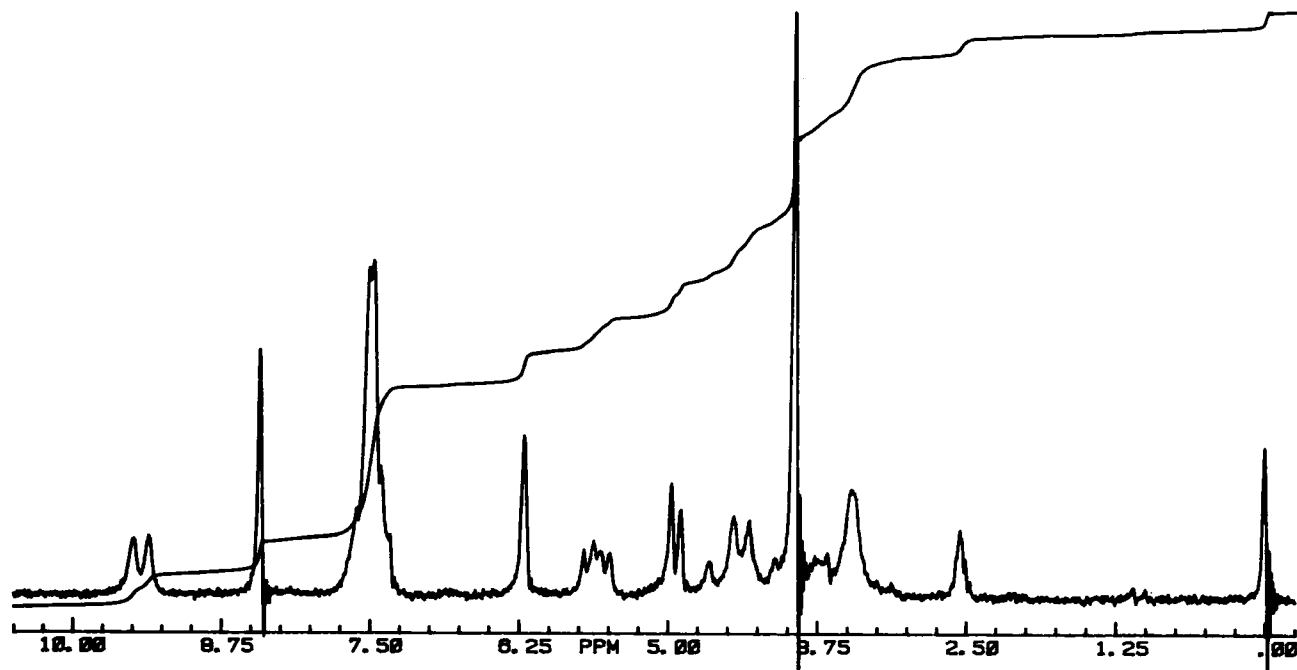
solvent

<sup>a</sup> Unless otherwise specified, each resonance represents a single proton. Coupling constants ( $J$ ) are in Hz.

<sup>b</sup> This resonance is superimposed by that due to moisture in the solid sample.

### 2.11.2 <sup>13</sup>C-NMR

The fully decoupled <sup>13</sup>C-NMR spectrum of cefamandole nafate in deuterium oxide is given in Figure 6. The spectrum was obtained on a Varian FT80-A instrument at



SWEEP TIME 250  
SWEEP WIDTH 1000  
SPIN RATE 45

DATE 2/12/00

CMPD CEFAMANDOLE NAFATE  
LOT# REFERENCE STANDARD  
CONC 100.2 MG./ML. IN DMSO-D6

Figure 5. Proton NMR Spectrum of Cefamandole Nafate

ambient temperature and using a 5mm sample tube. The data consist of 15,000 acquisitions of 16,384 data points over a 5000 Hz spectral width. The reference line is at 1348 Hz (67.4δ). Assignment of the resonances are as follows:

<u>Description<sup>a</sup></u>	<u>Assignment</u>
27.4	CH <sub>2</sub> (2)
34.8	N- <u>CH</u> <sub>3</sub>
37.1	CH <sub>2</sub> (3')
58.3	C(6)
59.5	C(7)
75.6	C <sub>6</sub> H <sub>5</sub> - <u>CH</u>
119.0	C(3)
128.4 (2C)	C(c), C(e)-aromatic
129.9 (2C)	C(b), C(f)-aromatic
130.6	C(d)-aromatic
131.7	C(4)
134.5	C(a)-aromatic
154.6	C(1)-tetrazole
162.8	<u>CHO</u>
164.6	C(8)
168.3	C(4')
171.8	<u>CO-NH</u>

<sup>a</sup> Chemical shift, δ, in ppm from TMS (0.0 ppm). Each resonance represents a single carbon unless otherwise stated.

### 3. Synthesis

D (-) Mandelic acid (I) is formylated to produce O-formylmandelic acid (II), which is then treated with excess thionylchloride to form D (-) O-formylmandeloyl chloride (III). Formylation of 7-aminocephalosporanic acid (IV) produces 7-formamidocephalosporanic acid (V), which is then treated with 1-methyl-1H-tetrazole-5-thiol, sodium salt (VI) to afford 7-formamido-3-(1-methyl)-1H-tetrazol-5-ylthiomethyl)-3-cephem-4-carboxylic acid (VII). Deformylation of (VII) yields 7-amino-3-(1-methyl-1H-tetrazol-5-ylthiomethyl)-3-cephem-4-carboxylic acid (VIII). The nucleus (VIII) is silylated with monosilylacetamide (MSA) and is then acylated



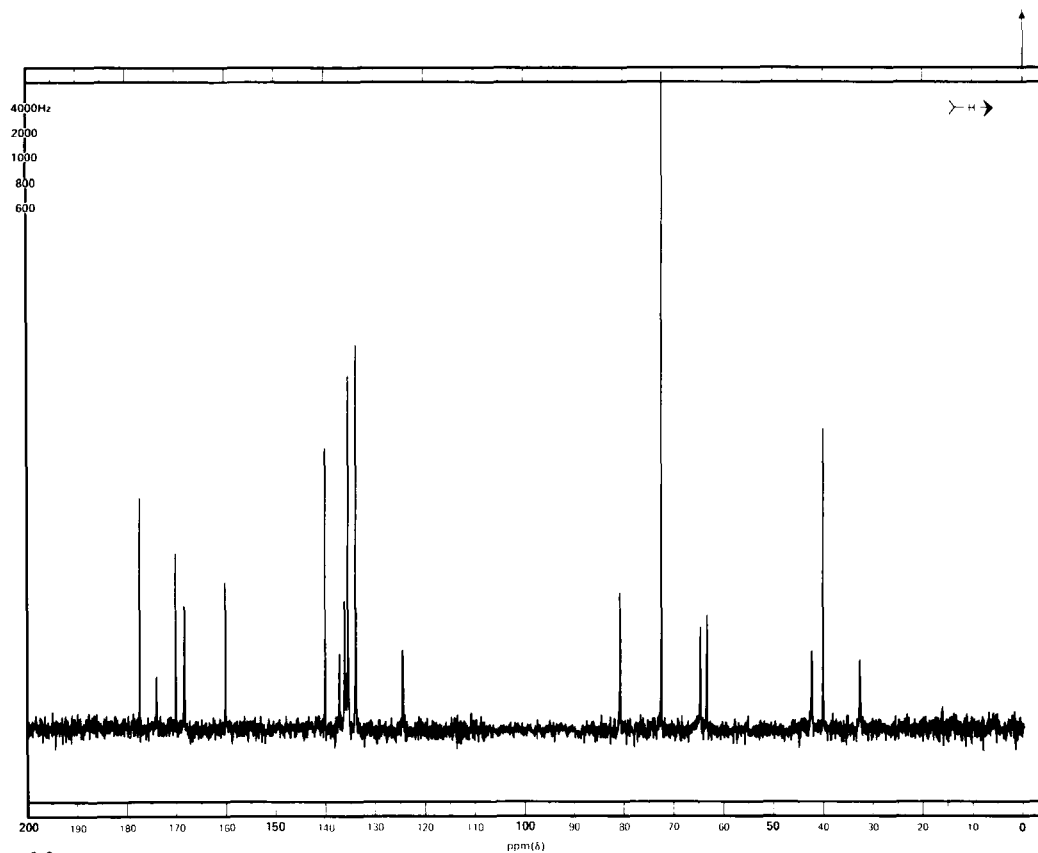


Figure 6.  $^{13}\text{C}$ -NMR Spectrum of Cefamandole Nafate

with O-formylmandeloyl chloride (III) to provide 7-(D-2-formyloxy-2-phenylacetamido)-3-(1-methyl-1H-tetrazol-5-ylthiomethyl)-3-cephem-4-carboxylic acid (IX). Alternatively, (IV) is acylated with (III) to produce 7-(D-2-formyloxy-2-phenylacetamido)-3-cephem-4-carboxylic acid (X). Addition of 1-methyl-5-thio-1, 2, 3, 4-tetrazole (XI) to (X) produces (IX). The sodium salt (XII) is produced by treating (IX) with sodium 2-ethylhexanoate in acetone. The flow diagram of the synthesis presented above (4-7) is shown in figure 7.

#### 4. Stability-Degradation

The ester function of cefamandole nafate is quite labile to nucleophilic attack by water or hydroxide ion in slightly acidic to slightly alkaline aqueous solutions in vitro (8), giving cefamandole (II) as the product (figure 8). Indelicato et. al. found that the formyl moiety of cefamandole nafate hydrolyzes with a half-life at 37°C which ranges from about 290 minutes at pH 5.5 to about 7 minutes at pH 8. In unbuffered solutions, the addition of bases such as sodium carbonate, ethanolamine and tromethamine produce rapid hydrolysis. For sodium carbonate, the fraction of cefamandole nafate which hydrolyzes is approximately equal to the number of equivalents of carbonate added per mole of cefamandole nafate and the hydrolysis reaches steady state in about 30 minutes or less for 0.28, 0.60 and 0.90 mole equivalents of carbonate. Ester hydrolysis is essentially complete within a few minutes when one mole of amine is added per mole of cefamandole nafate. Retention of chirality in the 7-D-mandelamido sidechain is observed for carbonate hydrolysis, which indicates cleavage of the acyl-oxygen bond. The hydrolysis of cefamandole nafate also occurs very rapidly in vivo with half-lives of 6-7 minutes and 10-17 minutes for dogs and humans respectively (9).

In artificially accelerated degradation studies (2, 10, 11), ester hydrolysis is observed in unbuffered solutions stored at 25, 37 or 60°C for 24 hours, in 0.1N hydrochloric acid at 25°C for 24 hours, in aqueous solutions (46°C) exposed to a high intensity UV light source for 64 hours, and in the reconstituted formulation. Hydrolysis of the solid sample varies with water content (11); no hydrolysis is observed for a dry (<0.1% water) sample after 2 months at 50°C (11), 24 hours at 100°C or 22 days at 60°C (10), but hydrolysis is observed in a wet sample heated for 3 weeks at 50°C (11). The 1-methyl-5-thio-1, 2, 3, 4-tetrazole (III) product is formed under the same conditions which produce ester hydrolysis. In addition to these

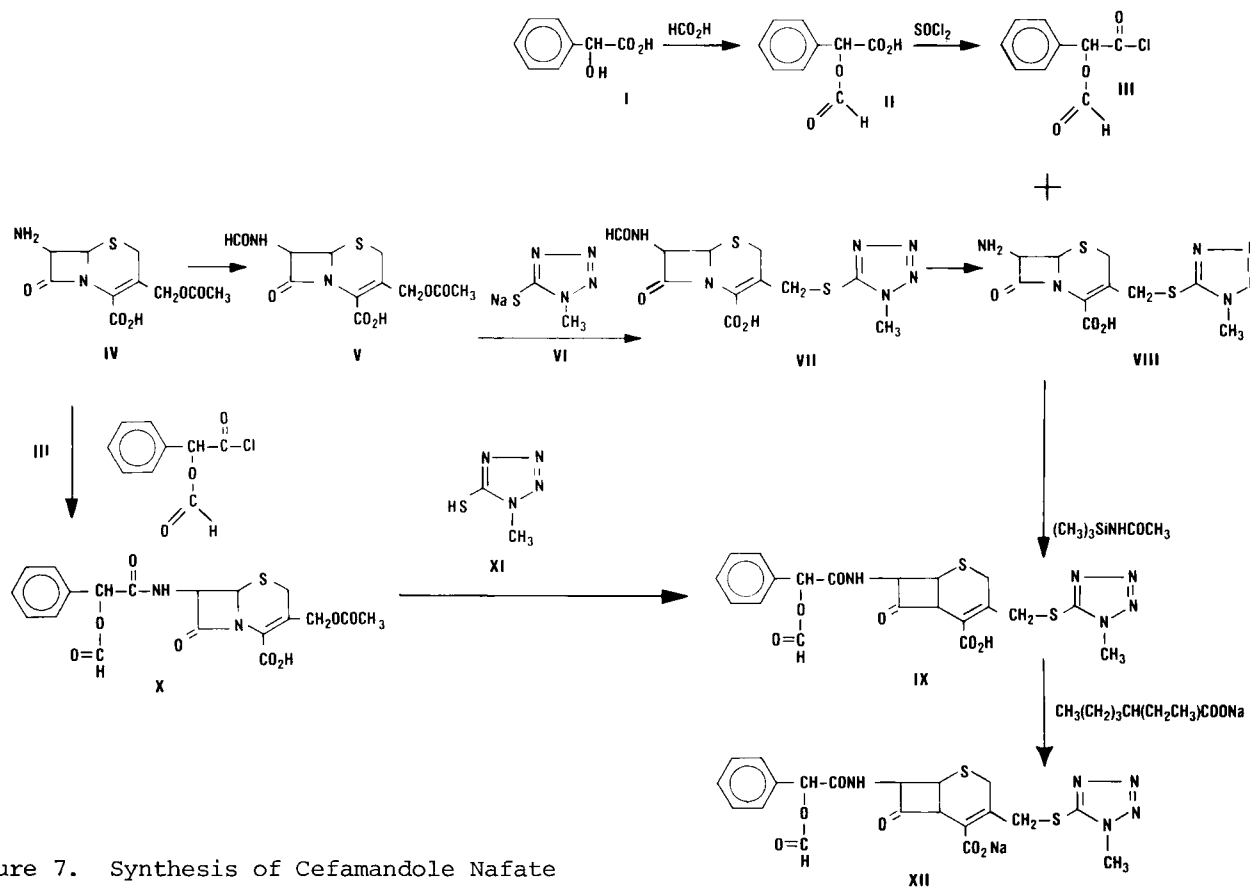


Figure 7. Synthesis of Cefamandole Nafate

reactions, other products can be formed. In neutral or unbuffered solutions, the nucleus which remains after loss of III may form the hydroxymethyl compound (IV) or, in slightly acidic solutions, dehydrate to the  $\alpha,\beta$ -unsaturated lactone (V). In extremely acidic conditions, the mandelic acid moiety (VI) is cleaved and the  $\beta$ -lactam system is destroyed. Strongly basic conditions produce mandelic acid and destruction of the  $\beta$ -lactam. This information is summarized in figure 8; the protonation of compounds I-IV and VI will depend upon pH.

## 5. Pharmacology, Bacteriology, Pharmacokinetics and Metabolism

### 5.1 Pharmacological Action

Cefamandole nafate is a parenteral cephalosporin antibiotic. In vitro, it is rapidly converted to cefamandole by hydrolysis of the formyl ester after dissolution (8). Because of rapid in vivo conversion of cefamandole nafate to cefamandole, the latter is the predominant circulating moiety after administration of cefamandole nafate to laboratory animals and humans (9). The calculated rate constant for hydrolysis of cefamandole nafate is higher in dogs than in humans, yielding  $t_{1/2}$  values of 6-7 minutes and 10-17 minutes respectively. Disappearance of cefamandole nafate from the plasma of dogs (due to hydrolysis and elimination) is slightly faster than from humans with a half-life ( $t_{1/2}$ ) of 4-6 minutes and 6-9 minutes, respectively.

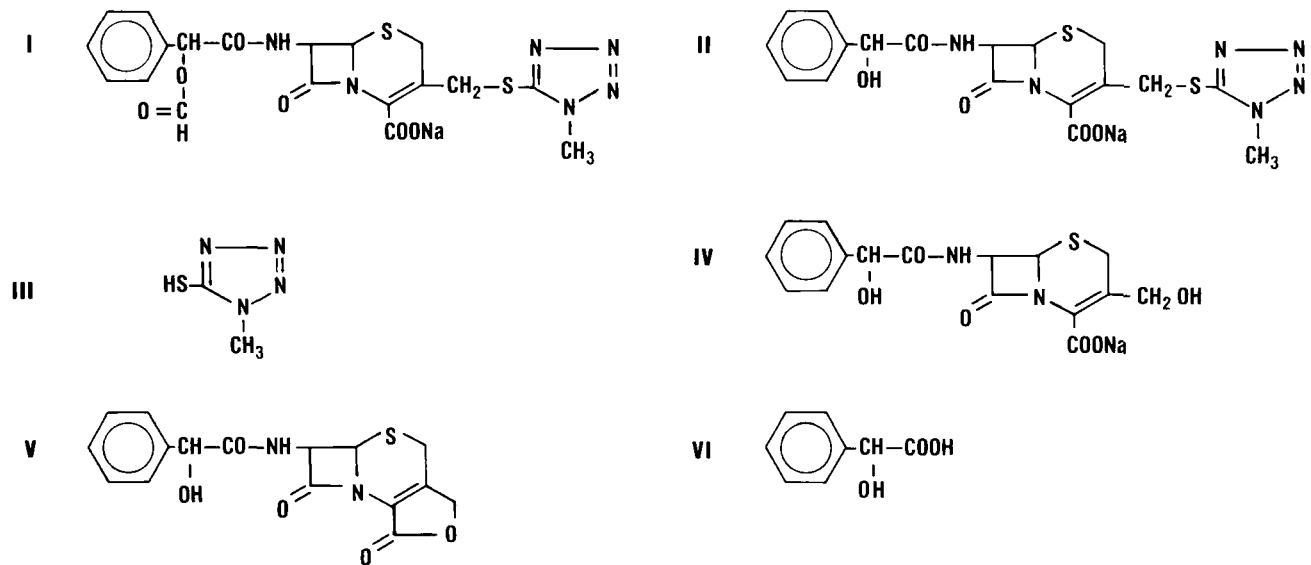
### 5.2 Antibacterial Activity

#### 5.2.1 In Vitro

In many conventional laboratory evaluation procedures, the in vitro antibacterial activities of cefamandole and cefamandole nafate appear virtually identical due to the hydrolysis of cefamandole nafate (2). Cefamandole is active in vitro against a variety of gram-positive and gram-negative microorganisms. In addition, it is active against a number of gram-negative aerobic organisms and both gram-positive and gram-negative anaerobes that have not traditionally been in the spectrum of the cephalosporins. Extensive in vitro studies have documented this expanded spectrum for cefamandole (13-26).

#### 5.2.2 In Vivo

The efficacy of cefamandole is identical to that of cefamandole nafate in treating experimental animal infections, indicating that rapid conversion of cefamandole nafate to cefamandole occurs in vivo (12).



Neutral: I  $\rightarrow$  II  $\rightarrow$  III + IV + V

Slightly Acidic: I  $\rightarrow$  II  $\rightarrow$  III + V

Slightly Basic: I  $\rightarrow$  II  $\rightarrow$  III + IV

Photolysis (aqueous): I  $\rightarrow$  II  $\rightarrow$  III + IV + V

Heat (dry): N.R.

Strongly Acidic: V  $\rightarrow$  VI + Other

Strongly Basic: III, IV  $\rightarrow$  VI + Other

Heat (Wet): I  $\rightarrow$  II  $\rightarrow$  III + IV + V

Figure 8. Accelerated Degradation of Cefamandole Nafate

### 5.3 Protein Binding

The protein binding of cefamandole is 74 percent when determined by an ultrafiltration method (27) and 67 percent with a range of 56-78 percent when measured by equilibrium dialysis (28). However, this data is obtained from an initial antibiotic concentration of 25mcg/ml and 20mcg/ml, respectively. Cefamandole appears to be rapidly dissociated from the serum proteins as indicated by its relatively short half-life and its rapid appearance in the urine (18).

### 5.4 Pharmacokinetics

#### 5.4.1 Serum Concentrations

After intramuscular administration of a 500mg dose of cefamandole to normal volunteers, the mean peak serum concentration is 13mcg/ml (18, 26, 29, 30). After 1g doses, the mean peak level is 25 mcg/ml (18, 26, 27, 30, 31). These peaks occur at 30-120 minutes. The decline of antibiotic concentration in the serum is biphasic with a rapid fall in the first two hours. Thereafter, levels decrease more slowly. Detectable concentrations are present for six to eight hours (18). Multiple-dose studies in patients given cefamandole intramuscularly show no evidence of accumulation (30, 32). Following intravenous doses of 1, 2 and 3g, serum concentrations are 139, 240 and 533mcg/ml at 10 minutes, respectively (18, 27, 29, 33). These concentrations decline to 0.8, 2.2 and 2.9mcg/ml at four hours. Intravenous administration of 4g doses every 6 hours produce no evidence of accumulation in the serum.

#### 5.4.2 Half-Life

The half-life after an intravenous dose is 32 minutes; after intramuscular administration, the half-life is 60 minutes (27, 28, 33, 34).

#### 5.4.3 Serum Clearance and Apparent Volume of Distribution (AVD)

A mean serum clearance of  $230 \pm 99$  ml/min./ $1.73m^2$  is found (27, 28) following intravenous administration of cefamandole. The AVD ranges from 10-67 percent of body weight. Following intramuscular administration of cefamandole the mean AVD is 17.11 liters, or 24 percent of the body weight (29), which is similar to the findings in the intravenous studies described above.

#### 5.4.4 Urine Concentrations, Excretion, and Renal Clearance

Cefamandole, in addition to being excreted by glomerular filtration, is also secreted by the renal tubules (32). Sixty-five to eighty-five percent of cefamandole is excreted by the kidneys after intramuscular injection of the drug, in the first 8 hours (18). The mean eight-hour urine concentrations are 254mcg/ml after 500mg doses and 1357 mcg/ml after 1g doses. Similar results are obtained by other investigators (27, 30). After intravenous administration of cefamandole, 75 to 100 percent is excreted in the urine in the first six to eight hours, and concentrations exceed 1000 mcg/ml with 500mg doses (27, 29). Probenecid slows tubular excretion and doubles the peak serum level and the duration of measurable serum concentrations. The renal clearance of cefamandole before and after administration of probenecid is  $302 \pm 60$  and  $80 \pm 14$  ml/min./ $1.73m^2$ , respectively (32, 34). In the presence of renal impairment, urinary excretion of cefamandole is slowed (32).

#### 5.4.5. Body Fluid and Tissue Concentrations

Distribution of cefamandole in body fluids and tissues following therapeutic doses of the antibiotic has been determined in bones and joints (35), gallbladder (36), interstitial fluid (37) and uterine tissue (38, 39). Tissue analysis gives primarily qualitative rather than meaningful quantitative data as to the presence or absence of an antibiotic in a particular body fluid or tissue. Therapeutic efficacy cannot be predicted by the level attained in a specific body fluid or tissue.

#### 5.5 Metabolism

A study of the metabolic fate of  $^{14}C$ -cefamandole in rats and dogs shows that after rapid in vivo hydrolysis of cefamandole nafate to cefamandole, the antibiotic is very resistant to metabolic degradation in both species (40). In dogs, cefamandole escapes metabolism and is eliminated as unaltered antibiotic almost exclusively by renal excretion. In rats, cefamandole is somewhat labile to metabolism. However, a major portion of the administered antibiotic is eliminated unchanged principally by renal excretion. Essentially all of the administered radiocarbon not eliminated by renal excretion is eliminated via biliary excretion. The fraction of radiocarbon dose remaining in the body of the rats after 24 hours amounts to less than 2%. The half-life of cefamandole in the blood of both species ranges from 30 to 42 minutes. Tissue level studies reveal no abnormal deposition of the antibiotic or metabolite in any tissue,

although all tissues examined contained concentrations of the antibiotic. The only tissues possessing significantly higher levels than that found in the blood are the kidney in both species and liver in dogs.

## 6. Method of Analysis

### 6.1 Elemental Analysis

<u>Element</u>	<u>Theory (%)</u>	
	<u>Sodium Salt</u>	<u>Free Acid</u>
C	44.53	46.53
H	3.34	3.70
N	16.40	17.13
O	18.73	19.57
S	12.51	13.07
Na	4.49	

### 6.2 Microbiological Assay

Cefamandole nafate is rapidly hydrolyzed to cefamandole in vivo (9) or in aqueous solutions of pH 5.5-8 (8). The in vitro activity of cefamandole nafate relative to that of cefamandole is different for some organisms when the assay conditions do not produce hydrolysis of cefamandole nafate (12, 41). Thus, a chemical hydrolysis is required prior to the microbiological assay in order to obtain results which are valid measurements of the bioactivity, and to avoid experimental difficulties due to partial hydrolysis during the assay. The microbiological assay is described for turbidimetric and agar diffusion methods. These assays are not specific for cefamandole nafate in the presence of impurities and/or degradation products. However, most contaminants will tend to have lower specific activity than cefamandole nafate so that a certain degree of selectivity is achieved.

#### 6.2.1 Turbidimetric Method

The turbidimetric assay is performed after hydrolysis to cefamandole, e.g., 15 minutes at room temperature with 0.87 moles of sodium carbonate per mole of cefamandole nafate followed by dilution with 0.1M phosphate buffer, pH6. The sample is further diluted to the reference concentration with 0.1M, pH6 phosphate buffer and added to medium #3 (42) inoculated with Staphylococcus aureus (ATCC 9144). Dose response concentrations are 0.02 to 1.0mcg cefamandole per ml of inoculated medium. The precision of the assay is about 2.5% as measured by the relative standard



deviation (RSD) of the assay (43).

#### 6.2.2 Agar Diffusion Method

For penicyclinder agar diffusion assays of raw materials or final dosage forms of cefamandole nafate, either S. aureus (ATCC 6538P) or Bacillus subtilis (ATCC 6633) may be used. With an agar plate system consisting of 10 ml of agar medium No. 2 (42) as base layer and 5 ml of agar medium No. 1 (42) as seed layer, dose response concentrations of 0.5 to 2.0 mcg of cefamandole nafate per ml are appropriate for both organisms. Sample preparation, hydrolysis followed by dilution in 0.1 M phosphate buffer (pH 6.0), is carried out in the same manner as for the turbidimetric assay. For assay of biological fluids, the B. subtilis assay is used. When sensitivity greater than 0.5 mcg per ml is required, a 5 ml single layer plate, medium No. 1, with dose response concentrations of 0.1 to 1.0 mcg per ml may be employed. Samples are not hydrolyzed since cefamandole nafate is converted to cefamandole in vivo (9, 12), but standard material must be hydrolyzed to cefamandole for preparation of standard curves.

#### 6.3 Iodometric Assay

Cefamandole nafate can be determined by an iodometric titration procedure similar to that applied to other cephalosporins (45). The  $\beta$ -lactam ring is hydrolyzed for about 10 minutes with alkali (0.2N NaOH), acidified (0.5N HCl) and allowed to react with iodine for about 5 minutes. All reactions are thermostated to 37°C. The difference in iodine uptake is measured between a blank (no sodium hydroxide added) and the sample. The RSD of this assay in an automated mode is about 1-2% (2). The measurement of iodine uptake is not specific for cefamandole nafate, especially in the presence of impurities and/or degradation products which contain the intact  $\beta$ -lactam system.

#### 6.4 Hydroxylamine Assay

The hydroxylamine assay is an alternate chemical assay procedure for cefamandole nafate (44). The method is based upon cleavage of the  $\beta$ -lactam by hydroxylamine to form a hydroxamic acid which is then reacted with acidified ferric ion to give a colored complex that can be monitored at 480nm. A blank correction for interfering non- $\beta$ -lactam chemical species which react with hydroxylamine is incorporated by adding the hydroxylamine to an acidic solution of the sample (the acid destroys all  $\beta$ -lactam entities). However, it is not possible to correct for interferences due to impurities and/or degradation products which contain an intact  $\beta$ -lactam.

## 6.5 Electrochemical Assay

The electrochemical assays rely on the reductive cleavage of the thioether linkage at the 3' position of the cephalosporin (2). This reduction occurs at the dropping mercury electrode (DME) with a half-wave potential of about -0.75V vs. SCE (saturated calomel electrode) for a 0.4mM sample concentration (0.2mg/ml) in a pH 2.4 McIlvaine buffer, and varies with concentration and pH (2). The electrochemical methods include controlled potential coulometry, DC or sampled DC polarography and differential pulse polarography.

### 6.5.1 Controlled Potential Coulometry

The controlled potential coulometric determination has been previously described (2). Coulometry is an absolute method in which the total charge consumed is measured during an exhaustive electrolysis. That is, no comparison to a standard is required (46). Thus, it is an important analytical tool for evaluation of purity of standard materials, but it is not normally used for routine assays. For cefamandole nafate, a potential of -0.875V vs. SCE is used and the reduction is performed at a stirred mercury pool electrode. The precision of this method applied to cefamandole nafate is approximately 0.95% as measured by the RSD (46). This assay measures all compounds which have functional groups that are reduced as easily or more easily than cefamandole nafate. However, the most common impurities and degradation products do not interfere (2).

### 6.5.2 DC and Sampled DC Polarography

DC and sampled DC (Tast) polarography can be applied to cefamandole nafate (2). These methods exhibit a precision of about 1.4-1.5% for the measurement of a sample versus a standard (2, 47). Their selectivity depends upon the difference between half-wave potentials of the various species. Since these methods can discriminate against more easily reducible as well as less easily reducible species, both are more selective than controlled potential coulometry (2, 48). These methods are more selective than microbiological, iodometric or hydroxylamine assays, but not as selective as the high performance liquid chromatographic assay. An automated, microprocessor controlled polarographic system has recently been described for this assay (49) and the linearity range investigated (47).

### 6.5.3 Differential Pulse Polarography

The differential pulse polarographic assay is the official chemical assay in the Code of Federal Regulations (45). It relies upon the same electrochemical

reduction process and achieves similar (or slightly greater) selectivity than DC polarography. The precision for this assay is about 1.2% for a sample measured versus a standard using the automated, microprocessor controlled system. The linearity and other characteristics of this assay have been recently described (47).

## 6.6 Chromatography

### 6.6.1 Paper Chromatography

Cefamandole nafate may be chromatographed on untreated Whatman No. 4 paper using a methylethyl ketone/water (92:8 v/v) developing solvent (40, 50). When about 30 ml of developing solvent is used and development time is 6-7 hours, the solvent front will run off the paper and the cefamandole nafate zone will move about 0.75 of the distance to the end of the paper. B. subtilis inoculated in an agar medium (6g peptone, 3g yeast extract, 1.5g beef extract, 20g agar dissolved in 1 liter of water and pH adjusted to 7.2) can be used for detection when approximately 1 mcg of sample is applied.

### 6.6.2 Thin Layer Chromatography

The  $R_f$  value is about 0.52 for cefamandole nafate when chromatographed on a silica gel 60 F254 thin layer plate developed by ethylacetate/acetone/glacial acetic acid/water (5:2:1:1 v/v/v/v) in a saturated chamber (2). The sample may be dissolved in water or in the developing solvent. The sample is visualized under short wavelength UV light (254nm) or under white light after exposure to iodine vapors. The Code of Federal Regulations 45 describes a continuous flow thin layer chromatographic (TLC) system for identification which employs a silica gel G thin layer plate developed by n-butanol/glacial acetic acid/water (4:1:1 v/v/v). This test uses a starch iodide/glacial acetic acid/0.1N iodine spray reagent for visualization. Other TLC systems and visualization procedures for cephalosporins are described by Marrelli (44).

### 6.6.3 High Performance Liquid Chromatography

A reverse-phase high performance liquid chromatographic (HPLC) assay may be performed using a C8 column with an isocratic eluting solvent (2% glacial acetic acid), 20% acetonitrile and 78% water, by volume) at a flow rate of 2 ml/minute (51). Samples are prepared in glacial acetic acid, detection is by UV absorbance at 254 nm. and the retention time for cefamandole nafate is about 9.5 minutes. This method exhibits a precision of about 2-3% (RSD). Cefamandole nafate may also be determined by ion-pair

chromatography using the C8 column with an eluting solvent of 25% acetonitrile, 2% acetic acid and 73% water containing 2% glacial acetic acid and 500 mg/ml of tetrabutylammonium dihydrogenphosphate (52). With a flow rate of 2.5 ml/minute, the retention time is about 15 minutes. Sample preparation and detection are identical to that described above.

#### 6.7 Analysis of Related Materials

Water may be determined by the Karl Fischer titration procedure. Other solvents, such as methanol, may be determined by a gas chromatographic (GC) assay. In the GC assay, the sample is dissolved into water and an internal standard solution such as ethanol is added. A 6 foot glass column packed with Porapak Q (60/80 mesh) and operated at 150°C with a helium carrier gas flow rate of 80 ml/minute gives retention times of approximately 1.1 and 2.4 minutes for methanol and ethanol, respectively. A Chromosorb 104 column may be used also. The flame ionization detector is used.

The hydrolysis product cefamandole and a potential impurity, 7-(D-2-formyloxy-2-phenylacetamido)-3-cephem-4-cephalosporanic acid (Compound X, figure 7) may be observed by TLC ( $R_f$  values of 0.46 and 0.58 respectively) (2) or determined using either of the HPLC methods described in section 6.5.3 (retention times of about 4.5 and 8.75 minutes for the first method and 6.25 and 13.5 minutes for the second method, respectively). Another possible impurity and degradation product 1-methyl-5-mercepto-1, 2, 3, 4-tetrazole, can be measured polarographically (2). It is oxidized at the DME in a pH 5.8 acetate buffer. The concentration of this species is proportional to the sum of the currents for the absorption prewave and the main wave (half-wave potentials about -0.20V and -0.05V vs. SCE, respectively).

#### 7. Analysis of Biological Samples

##### 7.1 Microbiological Assay

Serum and urine are assayed microbiologically using *B. subtilis* as the microorganism (18, 30, 40, 53-59). The microbiological assay is also used for determining the cefamandole levels in aqueous humor (59), ocular tissues (60-61), interstitial fluids, bile (55), pulmonary and subcutaneous tissue (58). A *klebsiella* strain is used as the indicator organism for assaying urine samples by an Autoturb (18).

##### 7.2 Liquid Scintillation Assay

$^{14}\text{C}$ -cefamandole is assayed by liquid scintillation procedure in metabolic and tissue distribution studies (40,

60, 61).

### 7.3 Chromatographic Assay

#### 7.3.1 Paper Chromatography

Cefamandole is assayed in biological samples on Whatman No. 4 paper developed with methylethylketone/water (92:8) for 7 hours to give a mobility of about 15cm from the point of application (40).

#### 7.3.2 Thin Layer Chromatography

Separation of cefamandole, cefamandole nafate and two metabolites is accomplished on silica Gel F plates developed with ethylacetate/acetone/glacial acetic acid/water (5:2:2:1) solvent. The  $R_f$  values are 0.55, 0.63 and 0.81, respectively (40).

#### 7.3.3 High Performance Liquid Chromatography

In studying the hydrolysis of cefamandole nafate to cefamandole, the biological samples are chromatographed on a column packed with Vydac reverse phase (30/44 $\mu$ m) and eluted with 20-25% acetonitrile in 0.1% aqueous acetic acid. At a flow rate of 2.0 ml/minute and monitoring the absorption at 254 nm, the retention time for cefamandole nafate and cefamandole are 4.4 and 7.2 minutes, respectively (9). Other HPLC work is performed on  $\mu$ Bondapak C18 column eluted with 30% methanol-0.01M sodium acetate, pH 5.2 at a flow rate of 2 ml/minute and monitoring at 270nm. The accuracy of the latter system is  $\pm 3\%$  and the reproducibility measurements yield a coefficient of variation of 4.6% (62).

## 8. Analysis of Pharmaceutical Formulations

The pharmaceutical formulation contains a buffering agent such that the reconstituted material has a pH between 6.0 and 8.0. Thus, when the material is reconstituted in water, it will be a mixture of cefamandole nafate and cefamandole (the hydrolysis product). The hydrolysis can be minimized by dissolving the sample in acetic acid or other acidic solvents. Somewhat different chromatographic, spectroscopic and physical characteristics will be observed on partially hydrolyzed samples compared to the corresponding data for the raw material.

The microbiological, iodometric, hydroxylamine, and polarographic (63) assays described in section 6 for the raw material are applicable to the formulation. The chromatographic methods will generally yield two zones (or peaks) corresponding to cefamandole nafate and cefamandole. Specifically, cefamandole gives a zone which is about 0.50 of the

distance to the end of the paper in the paper chromatographic system, a  $R_f$  value of 0.45 in the TLC system (2) and is observed in the HPLC systems as described in section 6.6.

#### 9. Acknowledgements

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The literature is surveyed through January 1980.

# CYPROHEPTADINE

*Hassan Y. Aboul-Enein and A. A. Al-Badr*

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## CYPROHEPTADINE

1. Description1.1 Nomenclature1.11 Chemical names

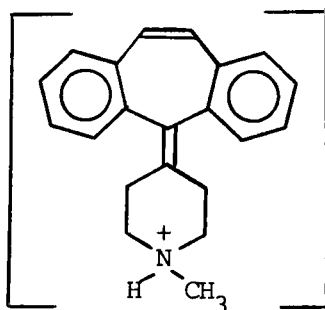
4-(5H-Dibenzo[a,d]cyclohepten-5-ylidene)-1-methyl piperidine; 1-methyl-4-(5H-dibenzo-[a,d] cycloheptenylydene) piperidine; 5-(1-methyl piperidylydene-4)-5H-dibenzo [a,d] cycloheptene; 1-methyl-4-(5-dibenzo [a,e] cycloheptatrienylydene) piperidine; 4-(1,2:5,6-dibenzocycloheptatrienylydene)-1-methyl piperidine.

1.12 Generic name

Cyproheptadine.

1.13 Trade names

Anarexol, Antegan, Nuran, Periactin, Vimicon, Cipractin, Peritol, Dronactine, Periactinol.

1.2 Formulae1.21 Empirical  $C_{21} H_{21} N$ 1.22 Structural

$Cl^- \cdot 1\frac{1}{2} H_2O$

1.23 Wiswesser line notation

L C676 BYJ BU DT6N DYTJ A (1)

### 1.3 Molecular weight

Anhydrous base 287.39, Anhydrous HCl 323.86  
Sesquihydrate HCl 350.89.

### 1.4 Elemental composition

C 87.76%, H 7.37%, N 4.87%.

### 1.5 Appearance, color, odor and stability

White to slightly yellow, crystalline powder that is odorless or practically odorless and has a slightly bitter taste; relatively stable in light, stable at room temperature and nonhygroscopic; the sesquihydrate is stable in air (2).

## 2. Physical properties

### 2.1 Melting point

The anhydrous form melts at about 250°. The sesquihydrate melts about about 162° (2). Crystals from absolute ethanol + ether, dec. 252.6-253.6°. Hydrochloride monohydrate crystal melts at 214-216° (3). Crystals from dilute ethanol (the base) melts at 112.3-113.3° (3).

### 2.2 Solubility

1 Gm in about 1.5 ml methanol, about 16 ml chloroform, about 35 ml alcohol and about 275 ml H<sub>2</sub>O; practically insoluble in ether (2).

### 2.3 Identification

The following tests are cited from the BP 1973 (4).

- a) The infrared absorption spectrum exhibits maxima which are compared to the authentic cyproheptadine hydrochloride.
- b) The light absorption, in the range of 230-350 nm, of a 2 cm layer of 0.0016% w/v solution in ethanol 95%, exhibits a maximum only at 286 nm, and extinction about one.
- c) A saturated solution yield the characteristic test for chloride.

The following are certain color tests (5) which are useful in the identification of cyproheptadine in micro amount :-

Reagent.	Color	Sensitivity
a) Sulphuric acid/formaldehyde.	Gray green	0.5 $\mu\text{g}$
b) Ammonium molybdate.	Blue green to green.	0.1 $\mu\text{g}$
c) Ammonium vanadate	Purple-brown.	0.5 $\mu\text{g}$

Furthermore, crystal tests can be used for the identification of the drug (5), for example, with ammonium thiocyanate solution, it gives branching needles (sensitivity 1 : 1000). With potassium iodide solution, dense roset and fans of rods (sensitivity 1 : 1000).

Yalcindag and Onur (6) had published a report which described the identification for some drug containing basic nitrogen including cyproheptadine through the microscopic appearance of crystals formed with a number of reagents and by some color tests.

## 2.4 Spectral properties

### 2.4.1 Infrared spectrum

The infrared spectrum of cyproheptadine base (Nujol mull) is given in Fig. 1, major band assignments are as follows :-

Frequency, $\text{cm}^{-1}$	Assignment.
3380, 3240	N-CH <sub>3</sub>
1590	Aromatic phenyl stretch.
1640	C=C at C <sub>10</sub> - C <sub>11</sub>

Other finger print bands has been assigned by Clarke (5), these peaks are :-

749, 797, 765 and  $776\text{cm}^{-1}$ , all of which are seen in the spectrum which is shown in Fig. 1. Further information with regard to the infrared spectrum of cyproheptadine is given in reference (1).

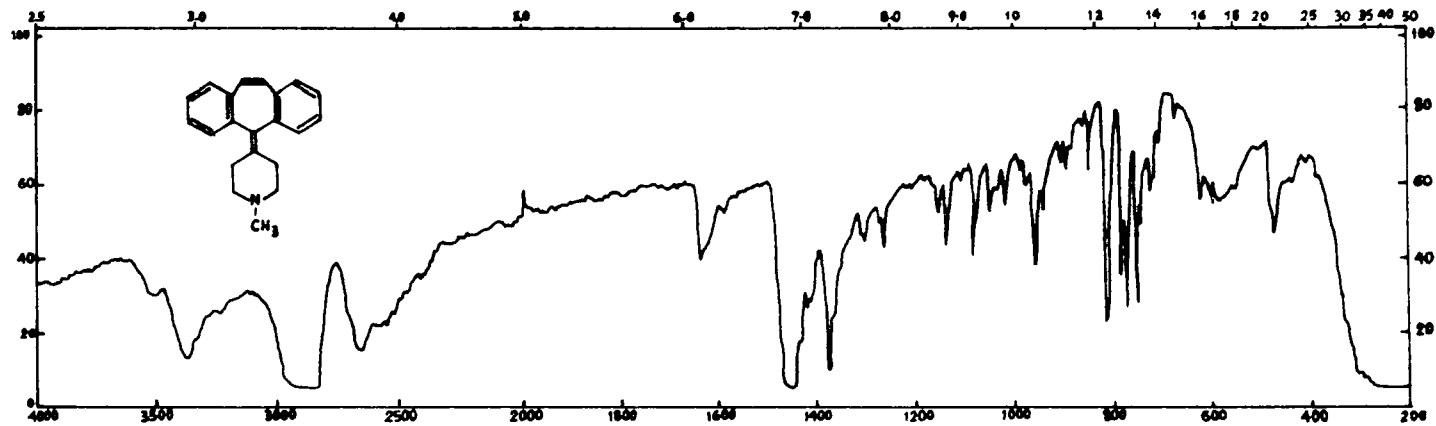


Fig. 1 - Infrared spectrum of cyproheptadine in Nujol mull.

#### 2.42 Ultraviolet spectrum

Cyproheptadine in methanol solution exhibits maxima at 224 nm, an inflexion at 240 nm and at 283 nm as shown in Fig. 2.

Clarke (5) reported the "ultraviolet absorption spectrum for cyproheptadine in 0.1N  $H_2SO_4$  to show maxima at 224 nm (E1% 1 cm 1656) and  $^{240}$  at 285 nm (E1% 1 cm 355), and an inflexion at 240 nm.

#### 2.43 Nuclear Magnetic Resonance Spectrum:

A typical NMR spectrum of cyproheptadine is presented in Fig. 3. It was obtained on a Varian T-60A NMR Spectrometer with TMS as the internal standard. The sample was dissolved in  $CDCl_3$ .

The following structural assignments have been made for Fig. 3:

Chemical shift (8)	Assignment.
- Singlet at 2.1	$N-CH_3$
- Broad complex multiplets between 2.27 and 3.47	8 protons of the piperidine ring system.
- Singlet at 6.87.	$CH = CH$ bridge at $C_{10}$ and $C_{11}$ .
- Multiplet centered at 7.23.	8 protons for the aromatic phenyl rings.

These data are in agreement with the data published by Englehardt et al. (7).

#### 2.44 Mass spectrum and Fragmentometry

The low resolution mass spectrum of cyproheptadine is shown in Fig. 4. It was obtained on a Finnigan 1015 L quadrupole mass spectrometer at an ionization potential of 70eV. The spectrum shown is obtained by direct insertion of cyproheptadine base. It shows a molecular ion  $M^+$  at  $m/e$  287 (Relative intensity 5.2%), a prominent peak at  $m/e$  96. The most important prominent ions are shown in Table 1.



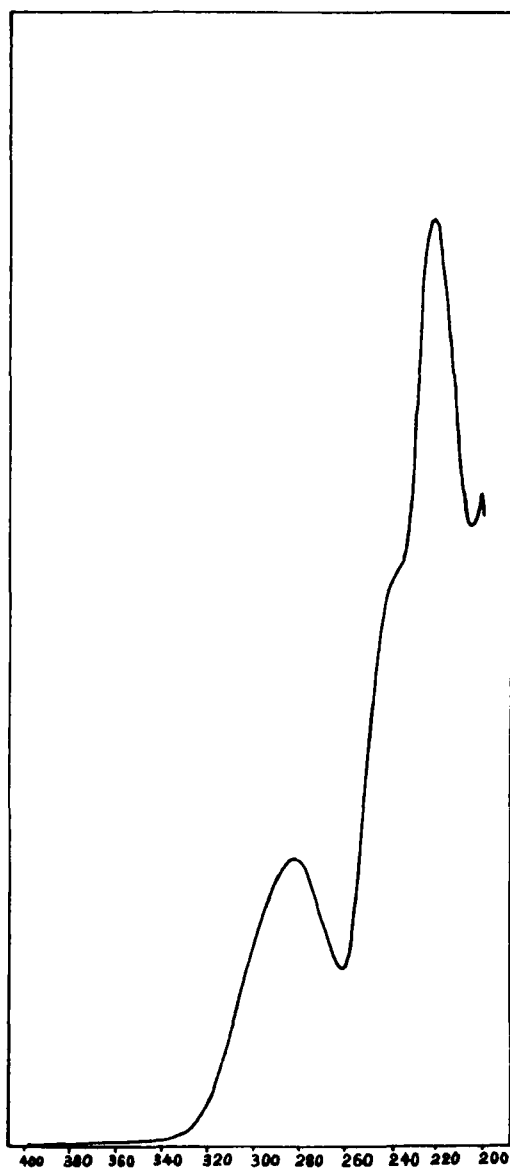


Fig. 2 - Ultraviolet spectrum of cyproheptadine in methanol.

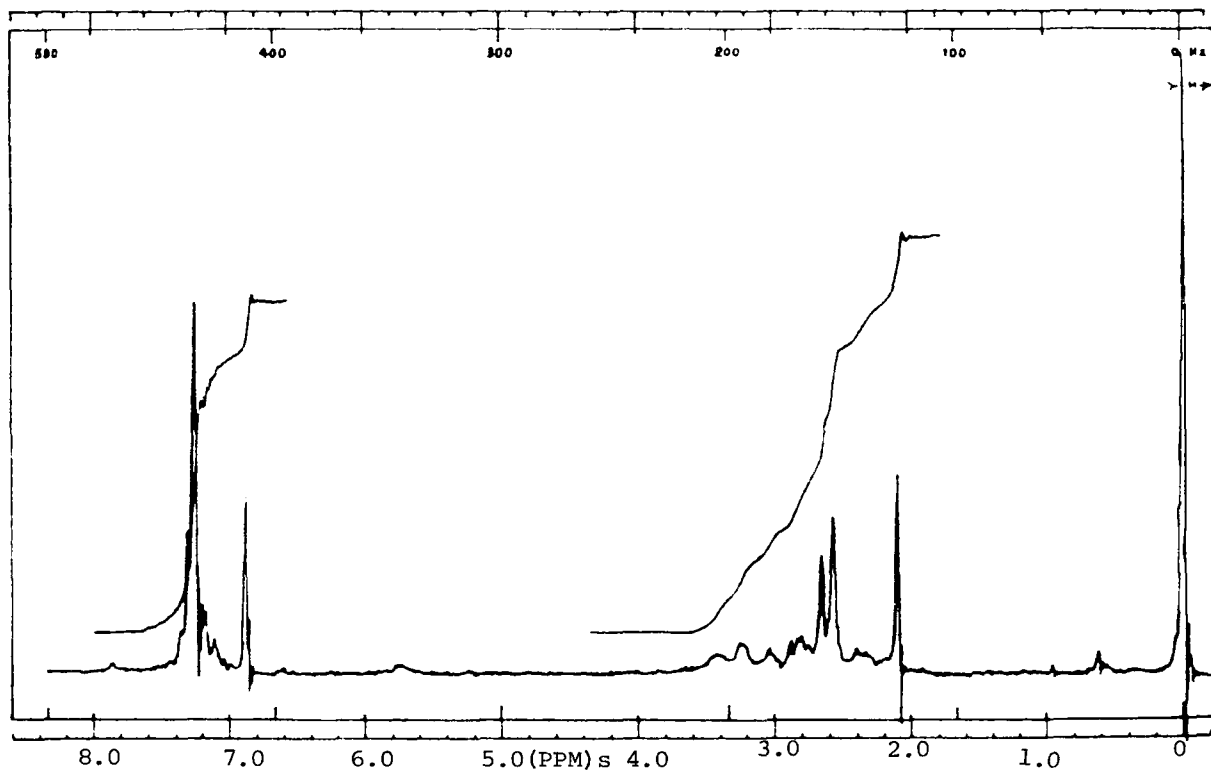


Fig. 3 - NMR spectrum of cyproheptadine in  $\text{CDCl}_3$  with TMS as internal standard.

11850 SCAN 101 SIGMA=8 RT=0.16 BAD=50.2100 100% 105100  
CPH-QU-225 3', 230'

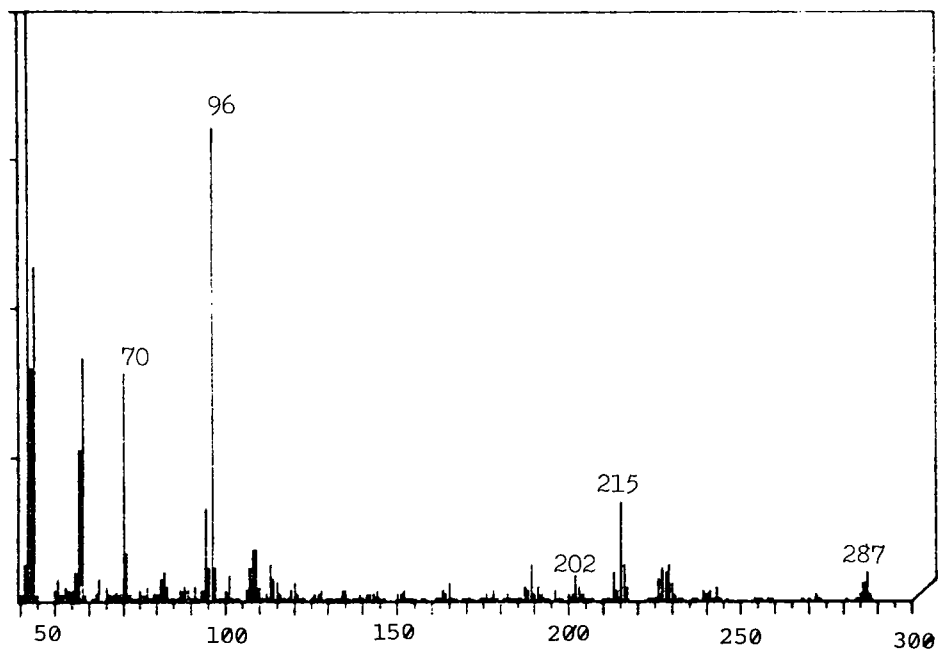
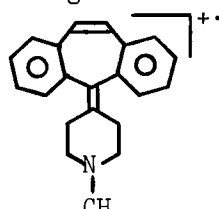
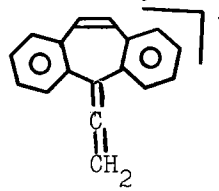
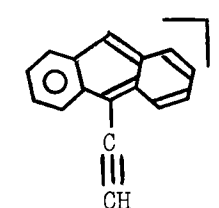
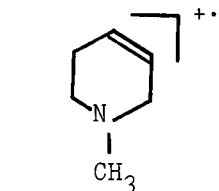
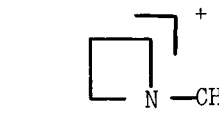
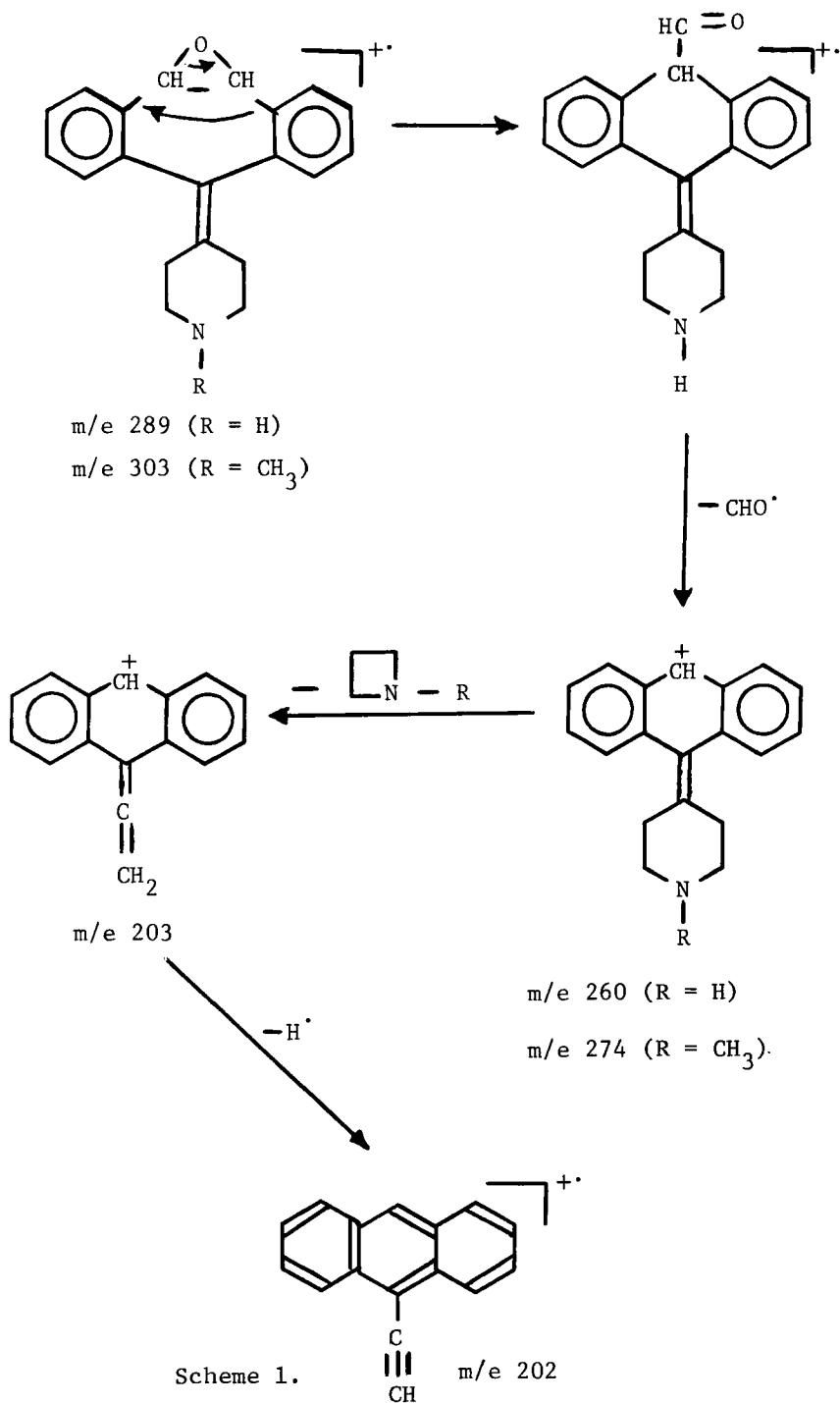


Fig. 4 - Mass spectrum of cyproheptadine (EI) determined by direct probe insertion.

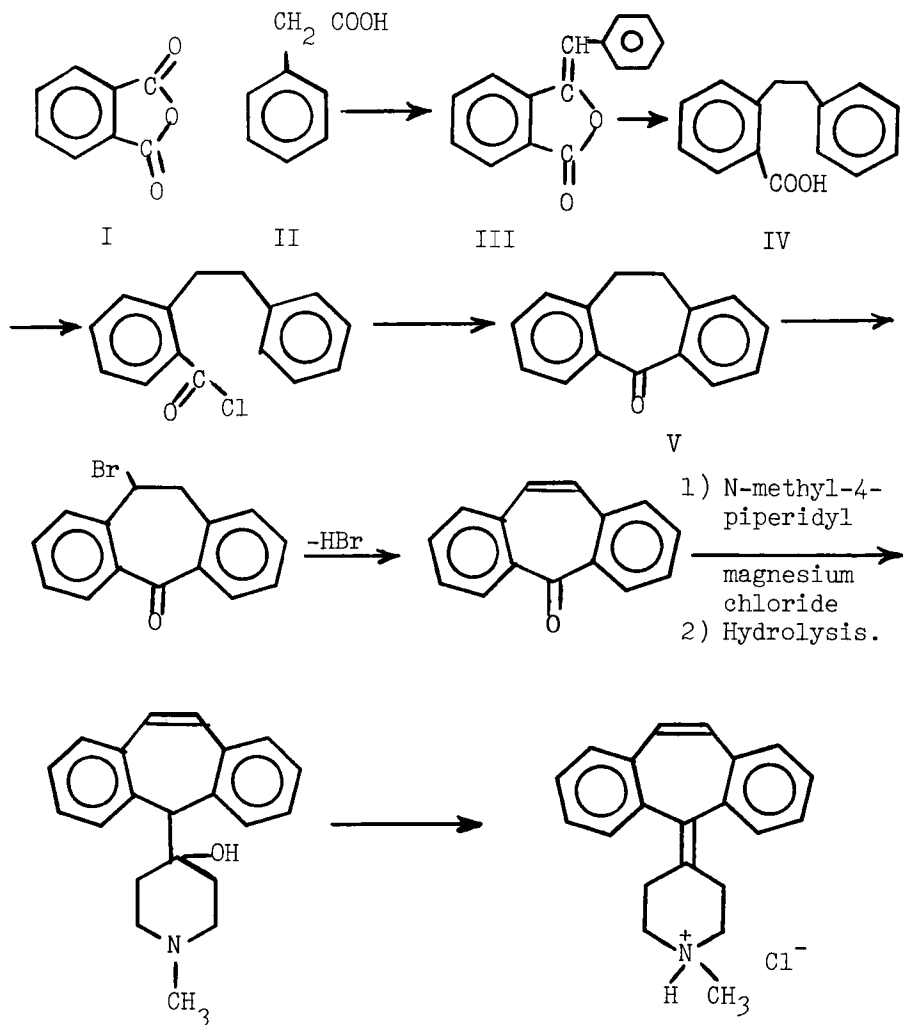
Fragment	Table 1	m/e	Relative intensity
		287	5.1
$M^+ - CH_3$		272	1.3
		215	17.4
		202	4.5
		96	80.2
		70	39.1

Frigerio *et al* (8,9) had discussed the fragmentation of some of the metabolites of cyproheptadine mainly cyproheptadine-10,11-epoxide, desmethyl cyproheptadine, desmethyl cyproheptadine-10, 11-epoxide. Frigerio *et al* (8) suggested a fragmentation pathway of these epoxide metabolites as shown on Scheme 1.

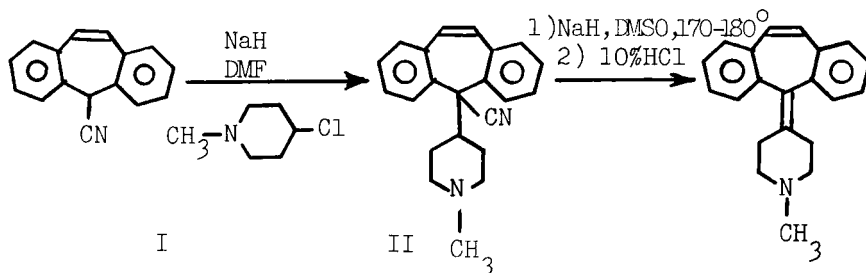


### 3. Synthesis

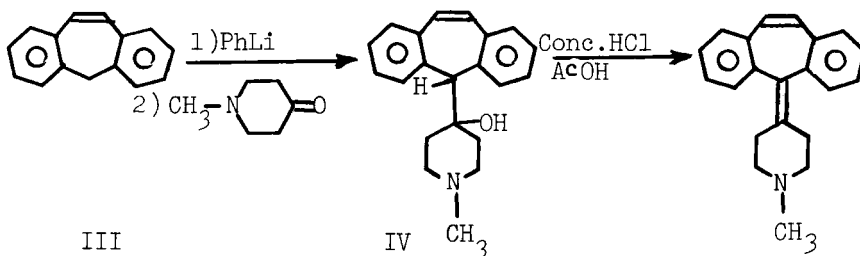
a) Phthalic anhydride (I) is reacted with phenylacetic acid (II) to form 3-benzylidenephthalide (III) which on isomerization and hydrogenation, gives 2-phenylbenzoic acid (IV). This is converted to its acid chloride which then undergoes condensation to close the 7-membered ring and give 10, 11-dehydro-5H-dibenzo [a,b] cyclohepten-5-one (V). Bromination at the 1- position followed by dehydrobromination introduces the 10, 11-double-bond. Grignardization of this ketone with 4-chloro-1-methyl piperidine followed by dehydration of the resulting carbinol yields cyproheptadine (base) which on reaction with equimolar quantity of hydrogen chloride, forms the hydrochloride salt (10).



- b) Converting 5-cyanodibenzo [a,d] cycloheptadine (I) to its corresponding piperidine derivative II which on treatment with sodium hydride, anhydrous dimethylsulphoxide at 170-180° then refluxed with 10% HCl, cyproheptadine was obtained in 87% yield (11).



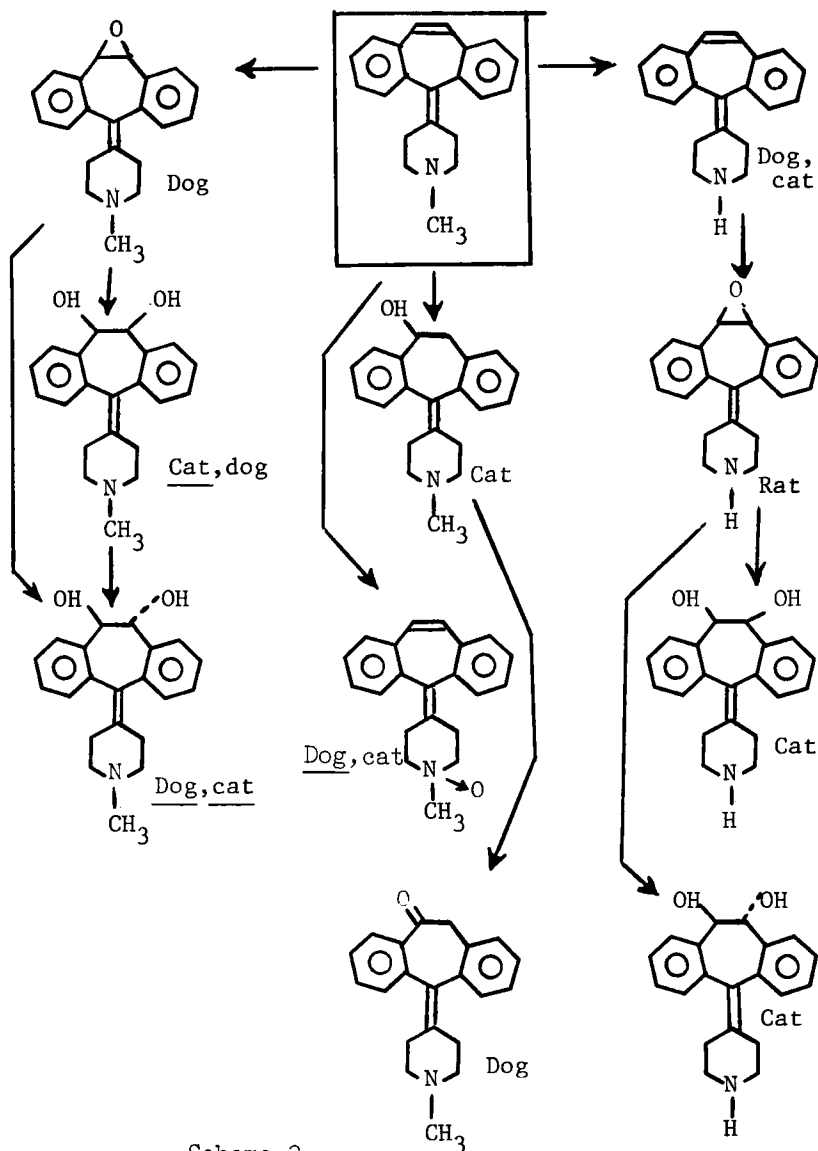
- c) Dibenzo [a,d] cycloheptene III was treated with phenyl lithuim in tetrahydrofuran and ether, the anion generated was reacted with 1-methyl-4-piperidone to give IV which was dehydrated by refluxing with acetic acid in concentrated HCl (11).



#### 4. Metabolism

The metabolism of cyproheptadine has been extensively studied in several species including humans.

Hucker *et al* (12) had published a report on the physiological disposition and urinary metabolite in the dog, rat and



Scheme 2.

Urinary metabolites of cyproheptadine identified in dog, rat and cat. Underlining indicates that the structure was a major metabolite in that species.



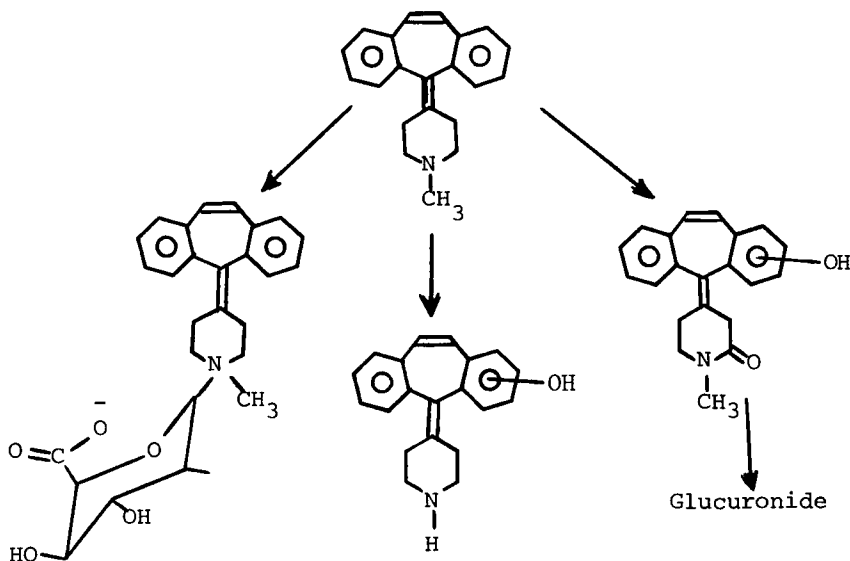
cat. It was found that, cyproheptadine was well absorbed and excreted almost equally in the urine and feces of these species. However, the plasma levels of the radioactivity were considerably higher in the dog than in the rat. About 17% of the dose was excreted in the dog bile in 6 hours. The urinary metabolites identified in the dog, rat and cat as, reported by Hucker et al (12), are shown in Scheme 2.

Rats excreted the drug almost entirely as the desmethyl cyproheptadine-10, 11-epoxide. This statement was substantiated by the study reported by Hintze et al (13).

Hintze et al (13) reported that after introducing a dose of  $^{14}\text{C}$  labelled cyproheptadine hydrochloride, the major metabolite in the rat urine was unconjugated, but the majority of the radioactive materials found in mouse and human urine were conjugated with glucuronic acid. The rat metabolite (the desmethyl cyproheptadine-10, 11-epoxide) accounted for 25% of 45 mg dose of the drug per kg. None of this epoxide was found in human.

The dihydrodiols which could arise from the 10, 11 epoxy metabolite were not found in the urine of the rat, mice and humans. The epoxide found in the rat urine reported to be unusually stable in the in vivo hydrolysis. Hintze et al (13) suggested possible implications of these results in the species-selected pancreatotoxicity of cyproheptadine in the rat. A detailed study on the  $\beta$ -cell toxicity of cyproheptadine is published by Rickert (14) and by Wold and Fischer (15). Frigerio et al (8) had identified cyproheptadine-10, 11-epoxide, desmethylcyproheptadine-10, 11-epoxide, and desmethylcyproheptadine in rat urine after administration of 40 mg/kg I.P. of the drug by mass spectrometry and confirmed their structure. Furthermore, Frigerio et al (9) in another report had identified the presence of the desmethylcyproheptadine-10, 11-epoxide in the urine of human volunteers. Porter et al (15) had reported a full study on the metabolism of cyproheptadine in humans. The metabolites identified are shown in scheme 3.

Aromatic ring hydroxylation followed by glucuronide conjugation, N-demethylation and heterocyclic ring oxidation were shown to occur in man. The principal metabolite, however, was identified as a quarternary ammonium glucuronide-like conjugate of cyproheptadine. They reported (16) no evidence for any metabolic changes at  $\text{C}_{10}$ ,  $\text{C}_{11}$  bridge



Scheme 3.

of the drug in humans. All metabolites seen in scheme 3, were identified by GLC, MS, NMR and IR spectrometric techniques.

## 5. Methods of Analysis\*

### 5.1 Spectrophotometric methods

#### 5.1.1 Colorimetric methods

Cyproheptadine had been determined in pharmaceutical formulation colorimetrically by Adamski (18). The ground tablets were extracted with chloroform, the extract was shaken with phosphate buffer and bromocresol green. The chloroform was re-extracted with 0.1N NaOH measuring the extinction at 615 nm referring the results to a calibrating graph.

---

\* Shapoval et al (17) published a report which include the physical, chemical and biological properties of the drug in tablets and the methods of its evaluation.

The error was reported to be  $> 1.2\%$ .

Beltagy et al (19) reported a method for the determination of 18 drugs in the free form and in various formulations colorimetrically using tropeolin 000. Cyproheptadine was included in that method of assay in which the drug was treated with tropeolin 000 in a pH 1.09 buffer. The complex formed was extracted with methylene chloride, the dye liberated by the acid addition was measured at 485 nm. The method gave results comparable to those obtained by the method of the B.P. 1973 (4).

#### 5.12 Ultraviolet Spectrophotometric method

This method has been adopted by the B.P. 1973 (4) for the assay of cyproheptadine tablets. The method cited in the B.P. 1973 depends on the measurement of the extinction of 1-cm layer of the alcoholic (95%) solution at a maximum at about 286 nm. The content of cyproheptadine hydrochloride is calculated taking 355 as value of E 1% 1-cm.

Demir and Amal (20) had published a similar procedure.

### 5.2 Titrimetric methods

#### 5.21 Nonaqueous titration

The B.P. 1973 (4) analyses cyproheptadine hydrochloride, free drug, by the non-aqueous titration using 0.1 N perchloric acid as a titration after the addition of mercuric acetate solution using crystal violet solution as indicator.

### 5.3 Chromatographic methods

#### 5.31 Counter-Current Distribution

Hintze et al (13) had isolated cyproheptadine and its epoxide metabolite from rat urine by the counter current distribution method. The pooled urine was adjusted to pH 8 and extracted several times with methylene chloride, the organic layer was concentrated in vacuo to 2 ml. After addition of

10 ml of 0.05M of phosphate buffer (pH 7.5), the remainder of the organic phase was evaporated. The buffer solution was placed in a 100 tube-counter current distribution apparatus and distributed between 0.05M phosphate buffer (pH 7.5) and benzene. After a 100 cycles, the solvents were decanted into glass-receiving tubes and aliquots of the benzene layer were removed for determination of radioactivity. The benzene layers of tubes 75-90 were combined and dried out over sodium sulfate. Mass spectrometry, and TLC were utilized to determine the purity of the metabolite and the unchanged drug that was isolated from rat urine.

Another method reported by Porter *et al* (16) for the counter current distribution. A gum isolated from human urine ingesting  $^{14}\text{C}$  cyproheptadine (5, 10, 11- $^{14}\text{C}$  4mg, 16  $\mu\text{Ci}$  per subject) after passing the urine through XAD-2 resin columns. The gum was subjected to fraction between water and butanol/benzene (1:1 v/v). Cyproheptadine and other metabolites were separated by this system and identified by TLC and GC.

### 5.32 Paper chromatography

Clarke (5) described a several solvent systems which are used for the identification of cyproheptadine as shown in Table 2.

Table 2

Solvent system	Visualizing agent	$R_f$
Citric acid : water : n-butanol	Ultraviolet, Iodo- platinate.	0.77
4.8g : 103 ml : 870 ml	(Weak reaction), Bro- mocresol green (weak reaction).	
Acetate Buffer (pH 4.58)	Ultraviolet Iodo- platinate.	0.22
Phosphate Buffer (pH 7.4)	Ultraviolet Iodo- platinate	0.00

### 5.33 Thin Layer Chromatography

Several reports had appeared in the literature concerning the tlc of cyproheptadine and its metabolites describing the separation and identification of cyproheptadine and its metabolites (5,8, 12, 13, 21, 22). The systems are given in Table 3. Ultraviolet light at 254 nm was used to detect the drug and its metabolites unless otherwise stated.

Hucker et al (12), Hintze et al (13) and Porter et al (16) had published the  $R_f$  values of cyproheptadine metabolites in several solvent systems which can be useful in clinical identification of the drug and its metabolites in biological fluids. Furthermore, Virgnoli et al (21) published a report on the identification of cyproheptadine among other drugs using Silica-gel as an absorbent in the following solvent systems :-

- A) Diethyl ether : acetone : diethylamine  
90 19 1
- B) Benzene : dioxane : diethylamine  
400 95 6

The chromatograms were sprayed by iodoplatinate followed by dilute  $H_2SO_4$  or 1% potassium permanganate in 5%  $H_2SO_4$  and iodoplatinate reagent.

### 5.34 Gas Liquid Chromatography

During the metabolic study of cyproheptadine in humans and other species, several gas chromatographic analyses were reported for the determination, identification and quantitation of cyproheptadine and its metabolites. The drug was chromatographed without derivatization. The gas chromatographic conditions are given in Table 4.

Table 3

Solvent system.			Absorbent	R <sub>f</sub>	Ref
Carbontetrachloride	: Methanol	: Diethylamine	Silica-Gel F254	0.60	8
85	: 10	: 5 v/v			
Acetone	: Ammonia		Silica-Gel GF 254	0.64	12
100	1				
Benzene	: Dioxane	: Ammonia	,, ,,	0.63	12
60	35	5			
Chloroform	: Methanol	: Acetic acid	,, ,,	0.72	12
47.5	47.5	5			
Chloroform saturated with	: Methanol		,, ,,	0.94	12
10 ammonia	1				
Benzene	: Dioxane	: Ammonia	,, ,,	0.90	12
10	80	10			
Benzene	: Ethanol	: Ammonia	,, ,,	0.73	12
95	15	5			

Table 3 continued.....

Solvent system.			Absorbent	R <sub>f</sub>	Ref
Methanol	: Acetone		Silica-Gel F 254	0.24	12
12	88				
n-Butanol	: Acetic acid	: Water	,, ,,	0.53	12
65	15	20			
Hexane	: Diethylamine		Silica-Gel GF	0.9	13
1	1				
Benzene	: Methanol	: Ammonia	,, ,,	0.86	13
50	50	1			
Benzene	: Dioxane	: Ammonia	,, ,,	0.91	13
1	8	1			
Strong ammonia solution		: Methanol	Silica-Gel G	0.50	5
1½	100				(Acidified Iodoplati- nate.

Table 4

Column.	Carrier Gas.	Column Temp. C	Refer-ences.
6 feet column packed with 3% OV-17 on acid-washed and silanized Gas-Chrom P	H <sub>2</sub>	Programmed from 150-250° at a rate of 5% per minute.	16
Glass tubing (1 m long and 4 mm i.d.) packed with 100-120 mesh Gas-Chrom Q and coated with OV-17.	N <sub>2</sub>	250	8
6 feet x ½-inch glass column packed with 1.5% OV-17/Gas-Chrom Q.	He <sub>2</sub>	238	12
5 feet x ½-inch o.d. glass column, 3% OV-225 on Supelcoport (80-100 mesh).	He <sub>2</sub>	225	13
5 feet x 4 mm i.d. glass column, packed with 2.5% SE 30 on 80-100 mesh Chromosob W AWHMDS.	N <sub>2</sub>	225	5

### 5.35 Partition Column Chromatography

Porter *et al* (16) have separated cyproheptadine metabolites by fractionation on columns packed with Cellex SE (H<sup>+</sup>) and on Bio-Rex 63 (H<sup>+</sup>) resin column.



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# DIBENZEPIN HYDROCHLORIDE

*Alfred Egli and Werner R. Michaelis*

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## 1. Introduction

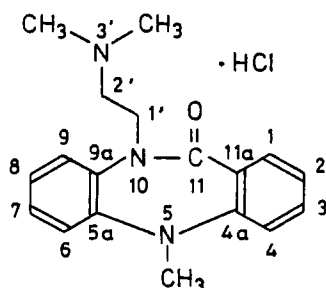
### 1.1 History

In 1959 and 1962, patent applications were filed for dibenzepin hydrochloride [1]. The drug substance shows remarkable histaminolytic and anti-anaphylactic effects [2]. According to clinical trials this antidepressant can be classified among the thymoleptic drugs between Imipramine and Amitriptyline [3, 4].

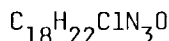
Dibenzepin hydrochloride is the active ingredient of the NOVERIL® dosage forms.

### 1.2 Name, Formula, Molecular Weight

Dibenzepin hydrochloride is 10-[2-(dimethylamino)ethyl]-5,10-dihydro-5-methyl-11H-dibenzo[b,e][1,4]diazepin-11-one, monohydrochloride



Molecular Formula:



Molecular Weight:

331.85

Chemical Abstracts Registry Number: 315-80-0

### 1.3 Appearance, Colour, Odour

Finely crystalline to crystalline, white or buff white powder; odourless or of weak, characteristic odour.

## 2. Physicochemical Properties

### 2.1 Elemental Analysis

Element	% Calculated	% Found
C	65.2	65.3
H	6.7	6.5
Cl	10.7	10.6
N	12.7	12.6
O	4.8	5.0

## 2.2 Spectra

### 2.21 Infrared

The IR spectrum in a KBr pellet as obtained on a PERKIN-ELMER 283 infrared spectrophotometer is presented in fig. 1.

The main characteristic bands are the following:

Wave number (cm <sup>-1</sup> )	Assignment
3100 - 2800	C-H stretching vibrations
2400 - 2560	N-H <sup>+</sup> stretching vibrations
1630	C=O stretching vibration
1600	C=O in-plane deformation vibration
775	C-H out-of-plane deformation vibration (1,2 disubstitution)

### 2.22 Ultraviolet

The UV spectrum in 0.1 N hydrochloric acid as obtained on a ZEISS DM4 spectrophotometer is presented in fig. 2. A maximum occurs at about 204 nm with a log molar absorptivity of 4.530, another maximum at about 220 nm with a log molar absorptivity of 4.458 and a shoulder at about 285 nm with a log molar absorptivity of 3.421.

### 2.23 Fluorescence

In 0.1 N hydrochloric acid the drug substance shows no fluorescence (excitation from 220 to 400 nm).

### 2.24 Proton Nuclear Magnetic Resonance

The PMR spectrum in deuterated dimethyl sulphoxide as obtained on a BRUKER HX-90-E spectrometer is presented in fig. 3. TMS served as internal standard. The characteristics of the spectrum are given in the following table:

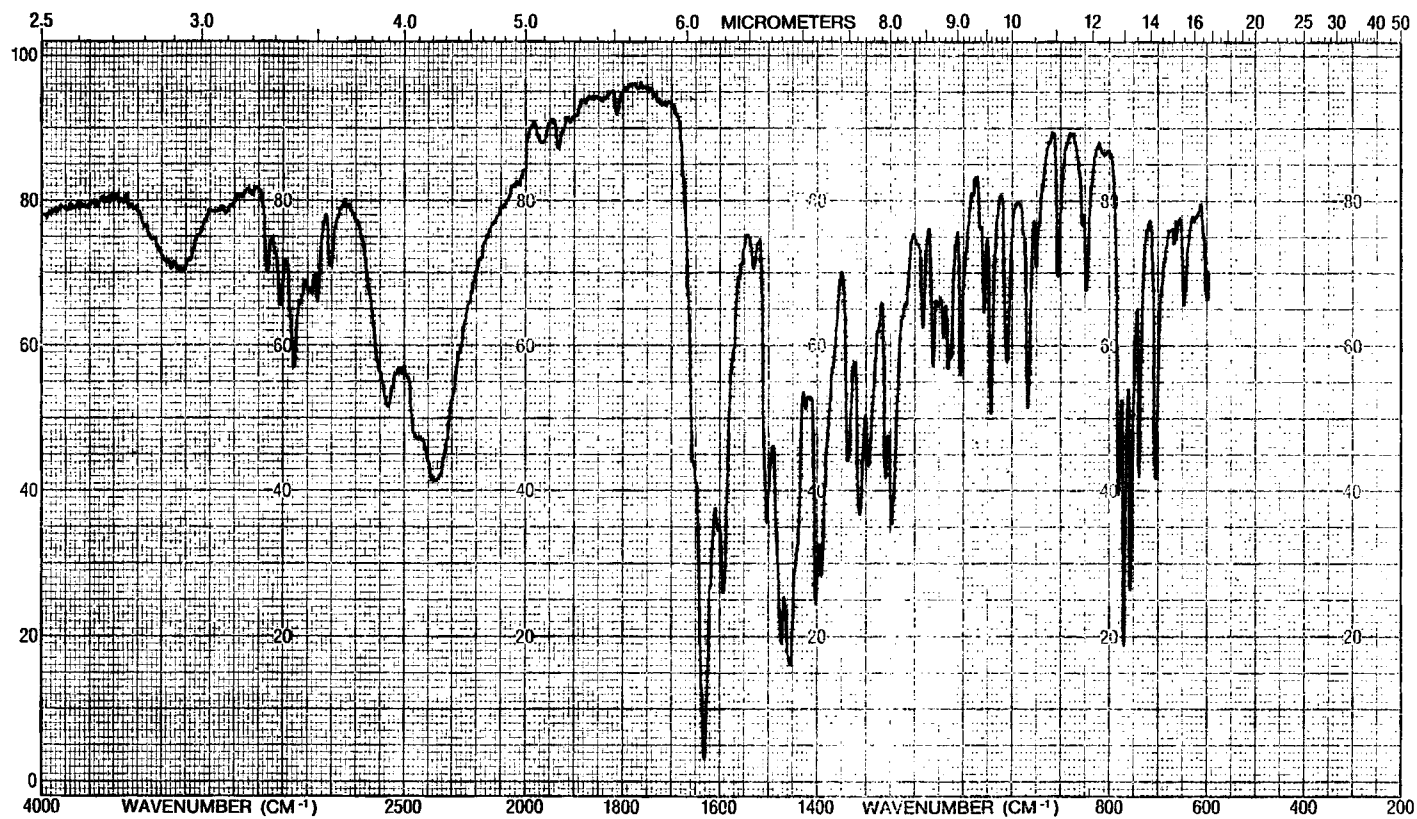


Figure 1: Infrared Spectrum of Dibenzepin Hydrochloride in a KBr Pellet.  
Instrument: PERKIN-ELMER 283.

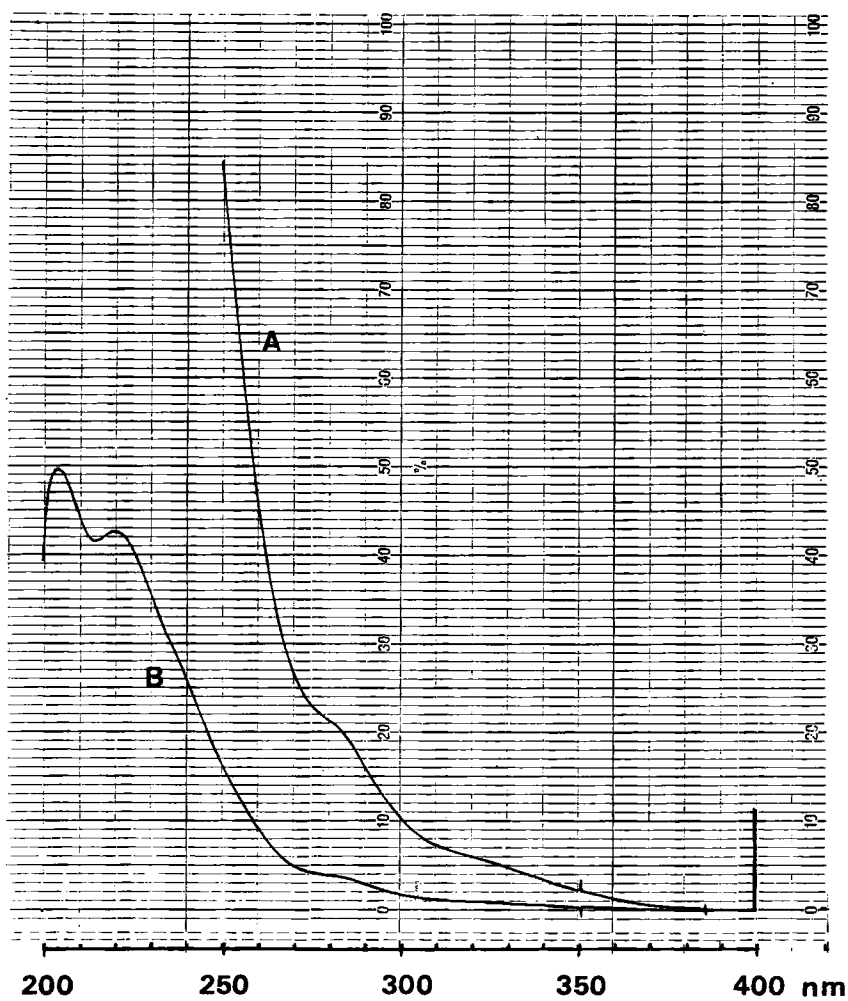


Figure 2: Ultraviolet Spectrum of Dibenzepin Hydrochloride in 0.1 N Hydrochloric Acid.

$C_A = 0.0505$  mg/ml;  $C_B = 0.0101$  mg/ml.

Instrument: ZEISS DM4.



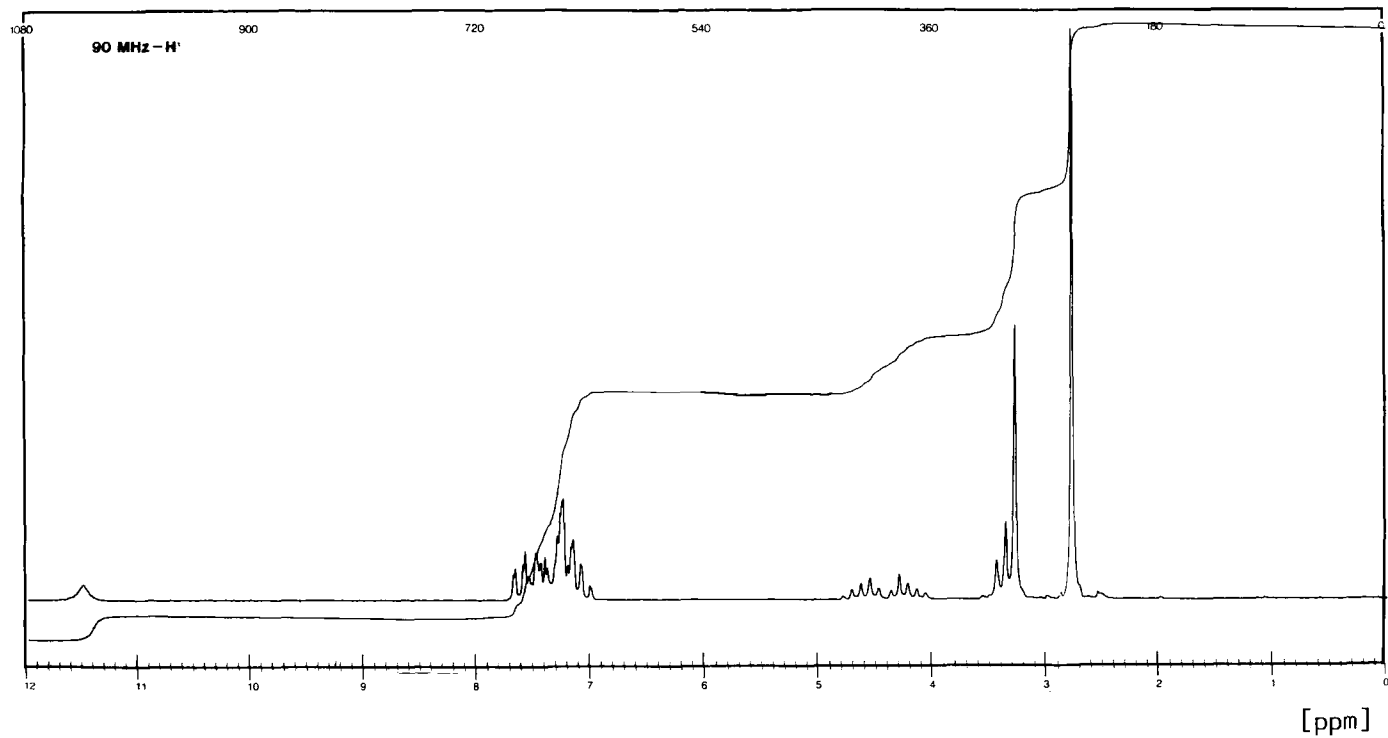


Figure 3: Proton Nuclear Magnetic Resonance Spectrum of Dibenzepin Hydrochloride in (CD<sub>3</sub>)<sub>2</sub>SO.

Instrument: BRUKER HX-90-E.

Chemical Shift [ppm]	Intensity	Multiplicity	Assignment
11.5	1 H	singlet (broad)	3'-H <sup>+</sup>
7.6	1 H	doublet of doublet	H-C1
7.4-7.5	2 H	multiplet	H-C3, H-C9
7.3	1 H	doublet of doublet	H-C6
7.15-7.3	3 H	multiplet	H-C4, H-C7, H-C8
7.1	1 H	triplet	H-C2
4.6	1 H	multiplet	H-C1'
4.2	1 H	multiplet	
3.35	2 H	triplet	H-C2'
3.3	3 H	singlet	5-CH <sub>3</sub>
2.8	6 H	singlet	3'-CH <sub>3</sub>

### 2.25 Carbon-13 Nuclear Magnetic Resonance

The C-13 NMR spectrum in deuterated dimethyl sulphoxide as obtained on a BRUKER HX-90-E spectrometer is presented in fig. 4. TMS served as internal standard. The assignment of the individual signals is given in the following table:

Carbon	Chemical Shift [ppm]	Carbon	Chemical Shift [ppm]
C-1	131.2	C-8	124.6
C-2	122.6	C-9	123.5
C-3	132.4	C-9a	134.8
C-4	116.4	C-11	168.0
C-4a	153.6	C-11a	126.5
5-CH <sub>3</sub>	36.6	C-1'	44.6
C-5a	148.3	C-2'	53.5
C-6	119.1	3'-CH <sub>3</sub>	42.0
C-7	126.3		

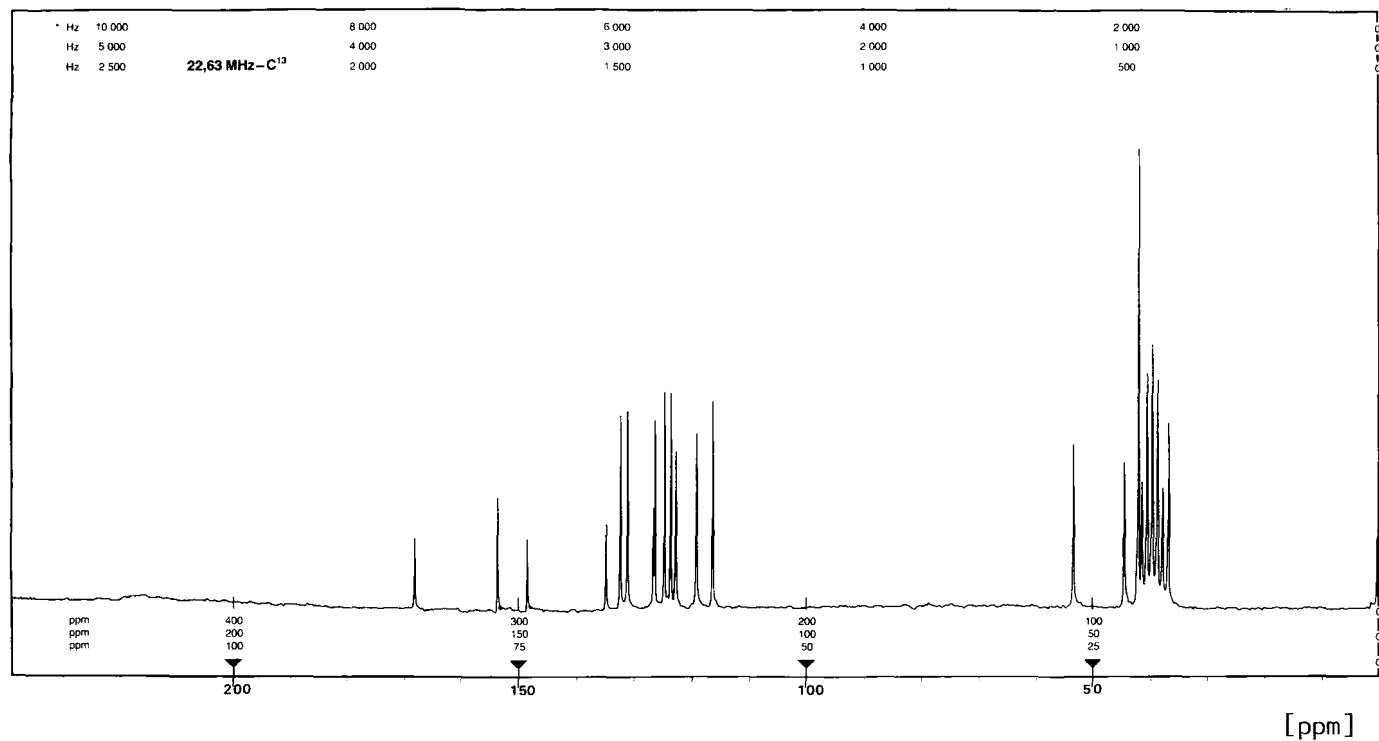
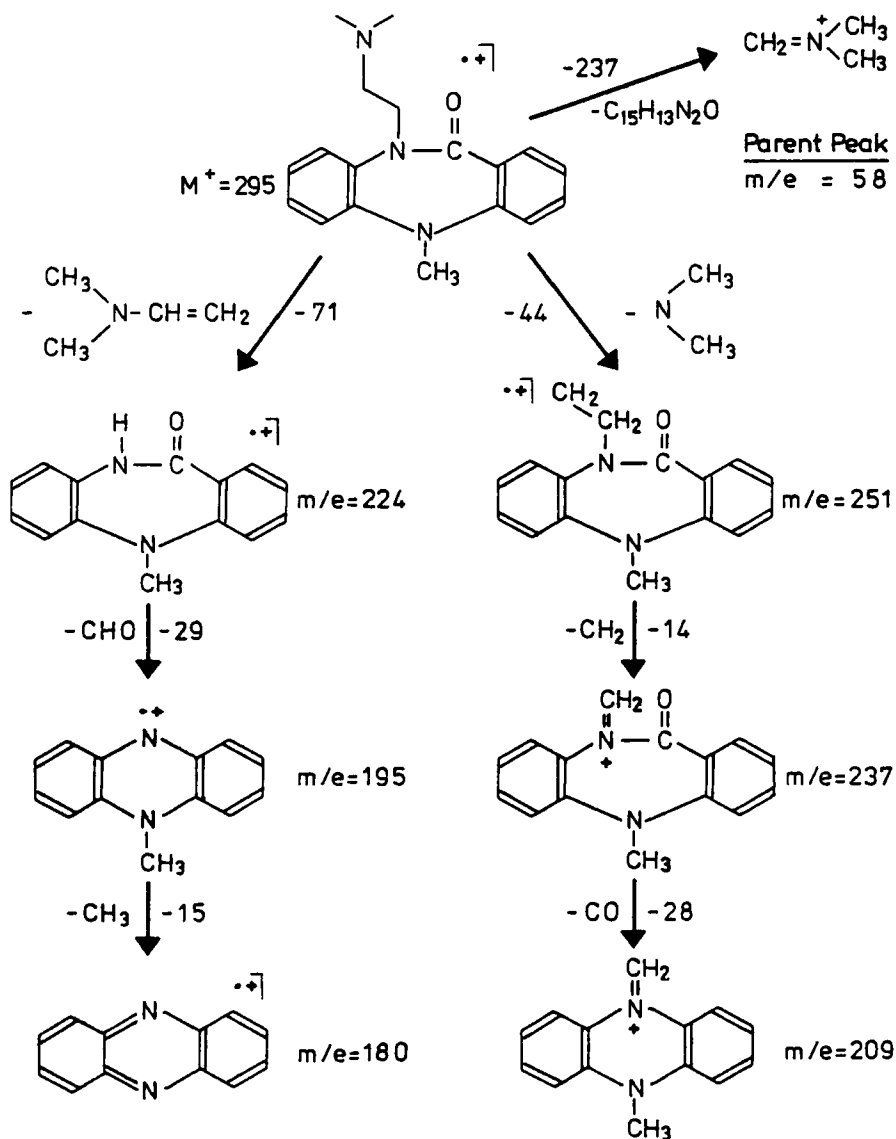


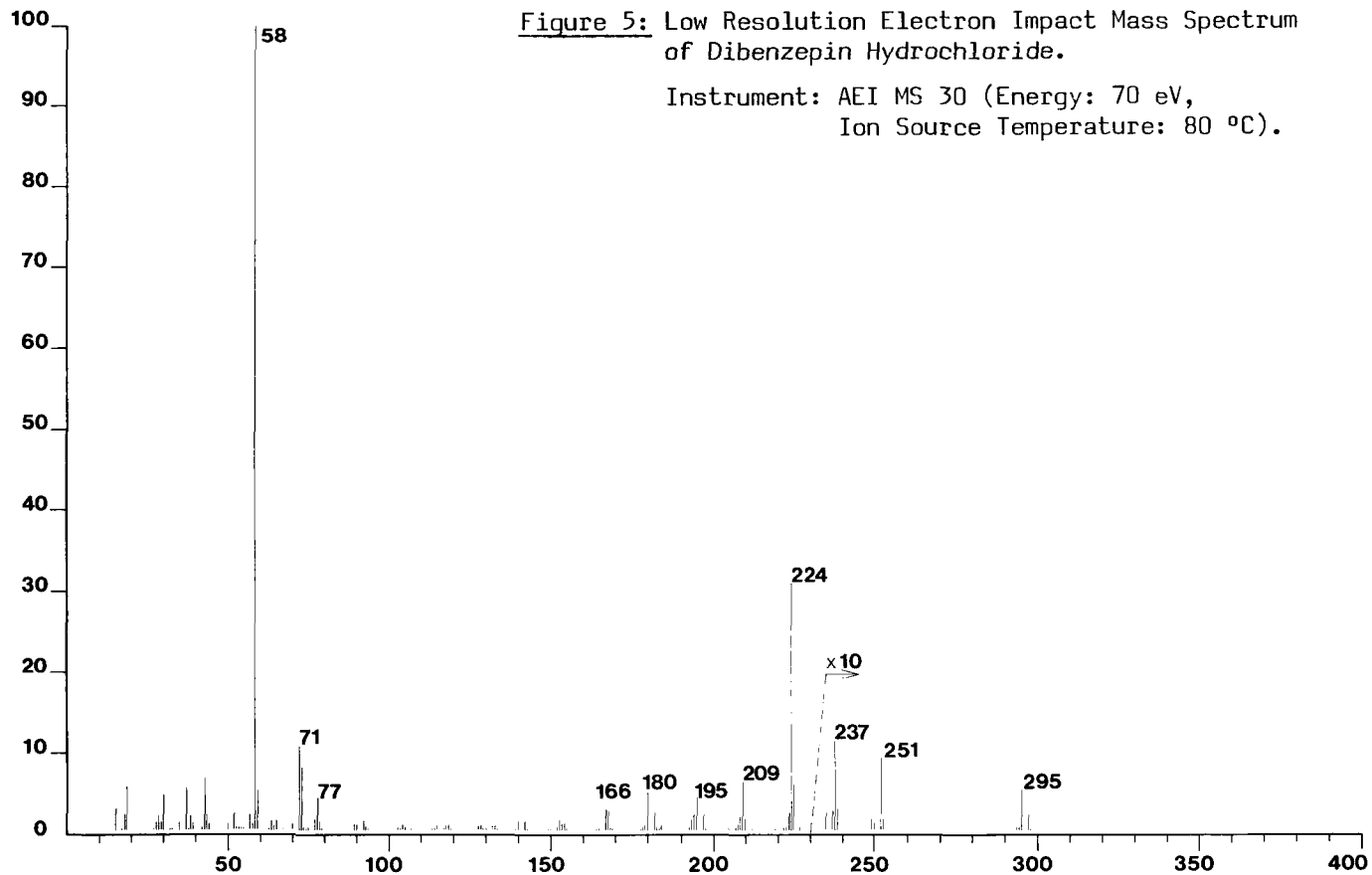
Figure 4: C-13 Nuclear Magnetic Resonance Spectrum of Dibenzepin Hydrochloride in (CD<sub>3</sub>)<sub>2</sub>SO.

Instrument: BRUKER HX-90-E.

## 2.26 Mass

The low resolution electron impact mass spectrum (70 eV) as obtained on a AEI MS 30 mass spectrometer using direct insertion probes at 80 °C is presented in fig. 5. The fragmentation pathways are as follows:





## 2.3 Crystal Properties

### 2.3.1 Melting Point

238 °C; the determination was carried out on a METTLER FP 1 (starting temperature 230 °C, heating rate 2 °C/min).

### 2.3.2 Polymorphism

So far no polymorphism has been observed by IR spectroscopy and differential scanning calorimetry.

### 2.3.3 Differential Scanning Calorimetry

The DSC thermogram, obtained with a PERKIN-ELMER DSC-2 instrument at a heating rate of 10 °C/min and in a nitrogen atmosphere, is shown in fig. 6.

The DSC curve shows only a sharp melting endotherm accompanied by decomposition or sublimation.

### 2.3.4 Thermogravimetry

The thermogravimetric curve, carried out on a PERKIN-ELMER TGS-1 thermobalance, is given in fig. 6. The sample temperature was raised at a rate of 10 °C/min maintaining a nitrogen atmosphere.

No loss of weight is observed until melting. A strong loss of weight is observed during the melting process.

## 2.4 Solubility

The solubility was determined in a variety of solvents equilibrated by vibration during 24 hours at 25 °C.

Solvent	Solubility in mg/g	Solubility in g/100 ml
water	more than 200	more than 20
methanol	more than 200	more than 20
ethanol	86	7.0
2-propanol	9.2	0.69
acetonitrile	14.4	1.1
acetone	2.2	0.17
ethyl acetate	0.5	0.04
chloroform	13.1	19.3
benzene	1.7	0.15
hexane	0.7	0.04

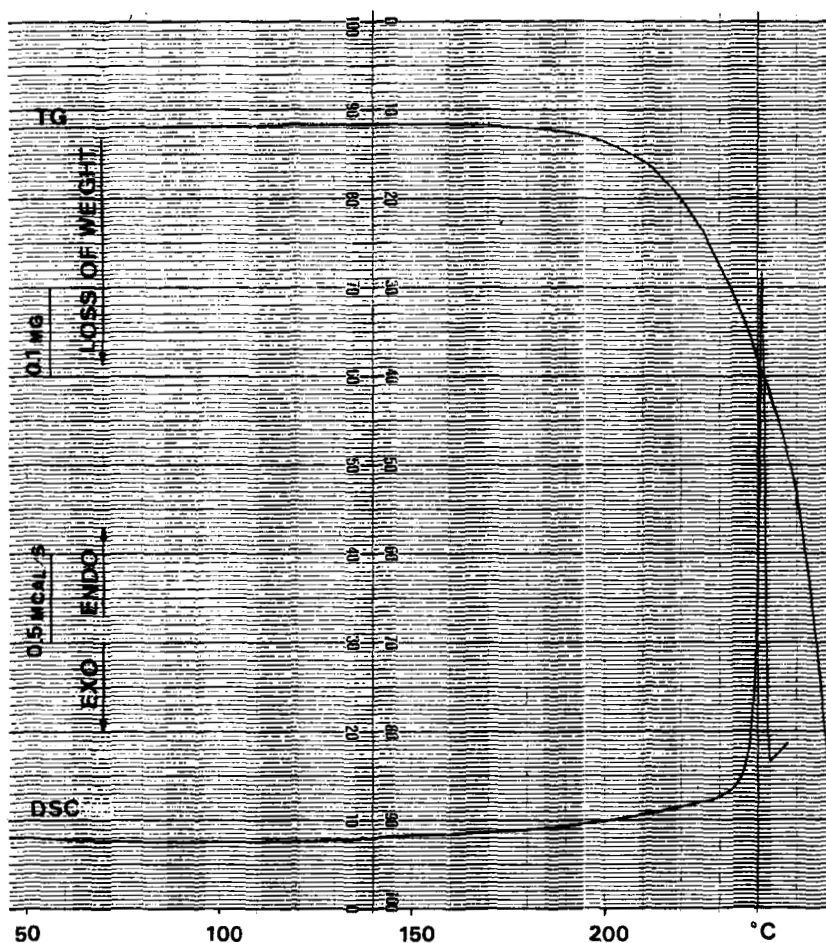


Figure 6: Differential Scanning Calorimetry and Thermo-gravimetry Curves of Dibenzepin Hydrochloride.

Instruments: PERKIN-ELMER DSC-2  
PERKIN-ELMER TGS-1  
(Heating rates 10 °C/min).

At  $22 \pm 2$  °C dibenzepin hydrochloride dissolves more than 2 % (w/v) in propylene glycol and ethanol 95 per cent, and more than 20 % (w/v) in ethanol 50 per cent; it is poorly soluble (0.056 % (w/v)) in n-octanol.

### 2.5 Dissociation Constant

Titration of a 0.003 M solution in water at 20 - 22 °C yielded as  $pK_a$   $8.25 \pm 0.05$  for the 3'-Nitrogen.

### 2.6 Partition Coefficients

The partition coefficients between simulated gastric fluid pH 1.2 (without enzyme) and n-octanol on one hand, and simulated intestinal fluid pH 6.8 (without enzyme) and n-octanol on the other, have been determined at  $37.0 \pm 0.5$  °C.

gastric fluid pH 1.2/n-octanol: 1 : 0.27

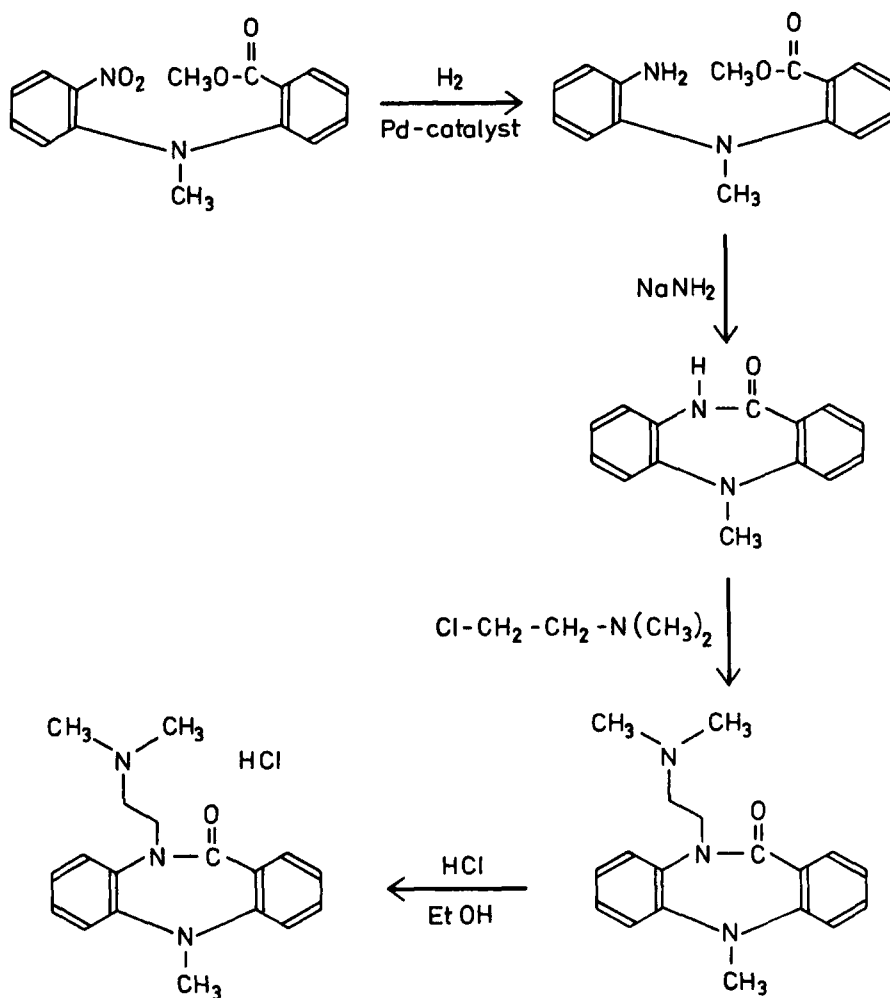
intestinal fluid pH 6.8/n-octanol: 1 : 18.3

## 3. Synthesis

Catalytic hydrogenation of 2-[methyl(2-nitrophenyl)-amino]benzoic acid methyl ester leads to the corresponding aminoester, 2-[(2-aminophenyl)methylamino]benzoic acid methyl ester, which is then converted by cyclization with a strong base (e.g. sodium amide) to the lactam 5,10-dihydro-5-methyl-11H-dibenzo[b,e][1,4]diazepin-11-one. Alkylation with 2-chloro-N,N-dimethylethanamine yields dibenzepin base, whose hydrochloride is formed by reaction with gaseous hydrochloric acid in ethanolic solution. Finally the product is recrystallized from ethanol [2].

The synthesis of C-14-labelled drug substance is described in [5].





#### 4. Stability

Dibenzipin hydrochloride is a very stable substance; a degradation could only be observed in acid solution under drastic conditions.

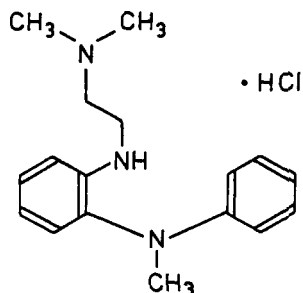
##### 4.1 Stability in Bulk

Samples stored in glass bottles for 15 years at 21 °C and for 8 years at 35 °C were investigated by TLC (3 systems): no degradation product could be detected (detection limit 0.05 %).

## 4.2 Stability in Solution

Dibenzepin hydrochloride is also very stable in solution: after refluxing a 10 per cent aqueous solution (pH 3.6) for 10 days only the active ingredient and no degradation product could be detected by TLC (3 systems, detection limit 0.05 %).

To degrade the active ingredient very drastic conditions are necessary: after refluxing a 10 per cent aqueous solution of pH 1 for 15 days about 10 % (w/w) of the following degradation product could be isolated and identified:



N-(2-(dimethylamino)ethyl)-N'-  
-methyl-N'-phenyl-1,2-benzene-  
diamine hydrochloride

No other degradation product could be detected.

## 4.3 Stability in Dosage Forms

Dibenzepin hydrochloride is marketed as NOVERIL® tablets, sugar-coated tablets, injection and concentrate intended for injection by intravenous infusion.

Since the active ingredient is stable in these dosage forms too, the shelf-lives in a temperate climate and in a hot climate of all these preparations are at least 5 years [6].

## 5. Biopharmaceutical Aspects

### 5.1 Pharmacokinetics [7]

The absorption, distribution and excretion of the C-14-labelled drug substance was investigated in the mouse after oral and i.v. administration of single doses and also after s.c. administration to the rabbit. In addition, radiochromatographic examinations of the brain extracts of mice, rats and rabbits were made.

In the mouse, orally administered dibenzepin hydrochloride was promptly and completely absorbed. After i.v. application, the radioactivity disappeared rapidly from the blood because the substance is taken up rapidly by the organs. The distribution of the activity in the various organs is independent of the mode of administration. The largest concentrations were found in the liver, kidneys, gall bladder, and the lungs.

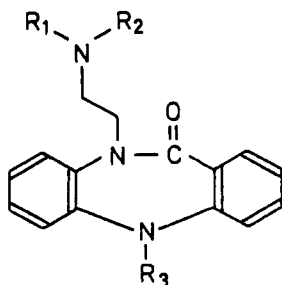
The drug substance is rapidly excreted. Half of the administered activity had already been excreted 5 h after oral administration and 100 min after i.v. application. After either oral or i.v. administration 80 % were excreted in the urine and 20 % in the feces.

The activity pattern in the rabbit was similar to that in the mouse: rapid and complete absorption and activity concentration in liver, kidneys, gall bladder, and lungs.

5 min after i.v. application, 2.6 % of the dose were found in the brain of the mouse and 1.6 % in the brain of the rat. 30 min after s.c. administration to the rabbit, 0.3 % were found in the brain. Between 1/2 and 4 h after administration to the rabbit the specific activity found in the bulbi olfact. was lower and that in the caudate nucleus was somewhat higher than in the rest of the brain.

Radiochromatographic examination showed that the activity found in the brain of mice, rats, and rabbits consisted mostly of unchanged dibenzepin. Besides this there were found the metabolite III (cf. 5.2) and two minor basic components of unknown structure, which together amounted to no more than 10 %.

## 5.2 Metabolism [8]



Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
I	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
II	H	CH <sub>3</sub>	CH <sub>3</sub>
III	CH <sub>3</sub>	CH <sub>3</sub>	H
IV	H	CH <sub>3</sub>	H
V	H	H	CH <sub>3</sub>
VI	H	H	H

The metabolites of orally administered dibenzepin hydrochloride excreted in the urine of man, dog and rabbit have been studied.

The compound is not retained in the body, but is rapidly metabolized and excreted in the urine. In all of the species, none of the metabolites was more toxic than the parent compound.

Man and dog excreted the unchanged compound and 5 demethylated derivatives (II-VI). Rabbits excreted the unchanged compound and the compounds II and III. In all three species, metabolites containing phenolic hydroxy groups were excreted. For the most part, these appeared in the urine as glucuronides. The dog excreted about 16 % of the administered doses as free basic metabolites. About 8 % were conjugated with glucuronic acid. 48 h after the last dose, no excretory products related to the drug substance were found in the urine.

Man excreted 20 - 30 % of the administered dose as free basic metabolites. The amounts present as the glucuronides were related to the dose. The formation of glucuronides was dependent on the dosage schedule; divided doses gave larger amounts than a single dose.

The rabbit excreted the drug principally as conjugates of the metabolites.

## 6. Acute toxicities

The acute toxicities ( $LD_{50}$ ) of dibenzepin hydrochloride were found to be: in the mouse, 22 mg/kg i.v. and 225 mg/kg p.o.; in the rat, 22.2 mg/kg i.v. and 220 mg/kg p.o.; and in the guinea-pig, 110 mg/kg p.o. [6].

## 7. Analytical Methods

### 7.1 Titration

Dibenzepin hydrochloride may be assayed in glacial acetic acid/acetic anhydride 1:1 (v/v) by titration with 0.1 N perchloric acid. The end point is determined potentiometrically using a glass/calomel electrode system.

The hydrochloric acid content of the dibenzepin hydrochloride is usually determined by titration with 0.1 N silver nitrate. The end point is detected potentiometrically using a silver/potassium sulfate electrode system.

### 7.2 Spectroscopic Methods

#### 7.21 Infrared

Infrared spectroscopy is utilized for identification purposes during the analysis of the drug substance (see 2.21).

#### 7.22 Ultraviolet

The drug substance can be assayed directly by measurement of the extinction at about 221 nm (maximum) or at about 280 nm (shoulder) in 0.1 N hydrochloric acid. The method is not specific, because by-products with the same chromophore are determined simultaneously. For the specific assay of the active ingredient it is necessary first to separate the by-products by thin layer chromatography and then to isolate the substance by elution from the silica gel of the plate with 0.1 N hydrochloric acid. The active ingredient is determined in the filtered 0.1 N hydrochloric acid.

#### 7.23 Colorimetry

In moderately acidic solutions dibenzepin hydrochloride readily forms ion pairs with methyl orange, which are extractable with chloroform. A procedure has been developed for assay with AUTO ANALYZER. Therein dibenzepin hydrochloride is allowed to react with methyl orange at pH 4.0. The resulting ion pair is extracted with chloroform and its concentration determined at 425 nm.

#### 7.24 Proton Magnetic Resonance

PMR spectroscopy may be used for identification of the drug substance (see. 2.24).

### 7.3 Chromatography

#### 7.3.1 Thin Layer Chromatography

The following systems can be used for the separation of by-products, degradation products, metabolites and excipients:

System	Stationary Phase	Mobile Phase
1	silica gel 60 F 254 (MERCK tlc plates, no 5715)	chloroform/methanol 1:1 (v/v)
2	silica gel 60 F 254 (MERCK tlc plates, no 5715)	ethyl acetate/ glacial acetic acid/ water 5:2:2 (v/v/v)
3	silica gel 60 F 254 (MERCK tlc plates, no 5715)	chloroform/cyclo- hexane/diethylamine 5:4:1 (v/v/v)
4	silica gel 60 F 254 (MERCK tlc plates, no 5715)	chloroform/cyclo- hexane/diethylamine 1:8:1 (v/v/v), twice developed
5	aluminium oxide F 254 (MERCK tlc plates, no 5713)	n-heptane/chloroform/ ethanol (95 per cent) 9:9:2 (v/v/v)

Visualisation is accomplished under UV light 254 nm and by spraying with Dragendorff's reagent. The  $R_{St}$  values are:

Substance	$R_{St}$ Value				
	System 1	System 2	System 3	System 4	System 5
dibenzepin hydro- chloride	1.0 (Rf 0.40)	1.0 (Rf 0.45)	1.0 (Rf 0.50)	1.0 (Rf 0.42)	1.0 (Rf 0.64)
degradation product*	0.90	1.15	1.20	1.75	1.15

\* N-(2-(dimethylamino)ethyl)-N'-methyl-N'-phenyl-1,2-benzene-diamine hydrochloride (for formula see 4.2).

System 4 is most suitable for the detection and semiquantitative determination of the degradation product.

The following reagents can be used for the visualisation of dibenzepin hydrochloride:

Reagent	Systems 1 - 4		System 5	
	Colour	Detection Limit [ $\mu\text{g}$ ]	Colour	Detection Limit [ $\mu\text{g}$ ]
Dragendorff's reagent(1)	brown	0.02-0.05	brown	0.05
iodine vapor	brown	0.2	brown	0.5
2,6-dichloro-p-benzoquinone-4-chlorimine- (modified Gibbs reagent)(2)	grey to greybrown	1.2	grey to green	0.2
Folin-Ciocalteus reagent(3)	blue	0.1	blue	0.05
sodium nitroprusside/ acetaldehyde	white to light violet	0.5	pink	0.5
potassium iodide/ hexachloroplatinic acid	pinkbrown to violet	0.5	pink	1
potassium dichromate/ sulfuric acid (40 per cent)	blue	0.2-0.5	brown	1

- (1) Dragendorff's reagent with consecutive spraying with a mixture of 20 ml hydrogen peroxide (30 per cent) and 10 ml of ethanol (95 per cent).
- (2) 90 - 110 mg 2,6-dichloro-p-benzoquinone-4-chlorimine are dissolved in a mixture of 25 ml chloroform, 25 ml ethanol 95 per cent and 3 ml dimethylformamide.
- (3) Sprayed with Folin-Ciocalteus reagent, MERCK no 9001 diluted with water 1:3 (v/v) and afterwards treated with ammonia gas.

The detection limits under UV light 254 nm are 0.1 - 0.2  $\mu\text{g}$ .

### 7.32 Gas Liquid Chromatography

The free base of dibenzepin hydrochloride can be determined by GC due to its volatility and its thermal stability. The conditions are the following:

Column: glass; length 2 m; internal diameter 2 mm

Stationary phase: Dexsil® 300, 1 % on Chromosorb® W, AW-DMCS (80 - 100 mesh)

Mobile phase: nitrogen, flow rate 35 ml/min

Temperatures: injector: 250 °C

detector: 300 °C

column: 200 °C for 2 min; temperature

gradient: 8 °C/min; final temperature: 300 °C.

Fig. 7 shows a gas chromatogram of a dichloromethane solution of dibenzepin spiked with the degradation product and octacosane as an internal standard.

### 7.33 High Performance Liquid Chromatography

A HPLC system has been developed on reversed phase (octylsilanised silicagel column) for assay and purity testing of dibenzepin hydrochloride.

#### HPLC-conditions

Stationary phase: LiChrosorb® RP-8 (MERCK), 10 µm in stainless steel, 25 cm x 4.6 mm i.d.

Mobile phase: isocratic: methanol/1 per cent ammonium carbonate solution 65:35 (v/v)

UV detection: at 221 nm

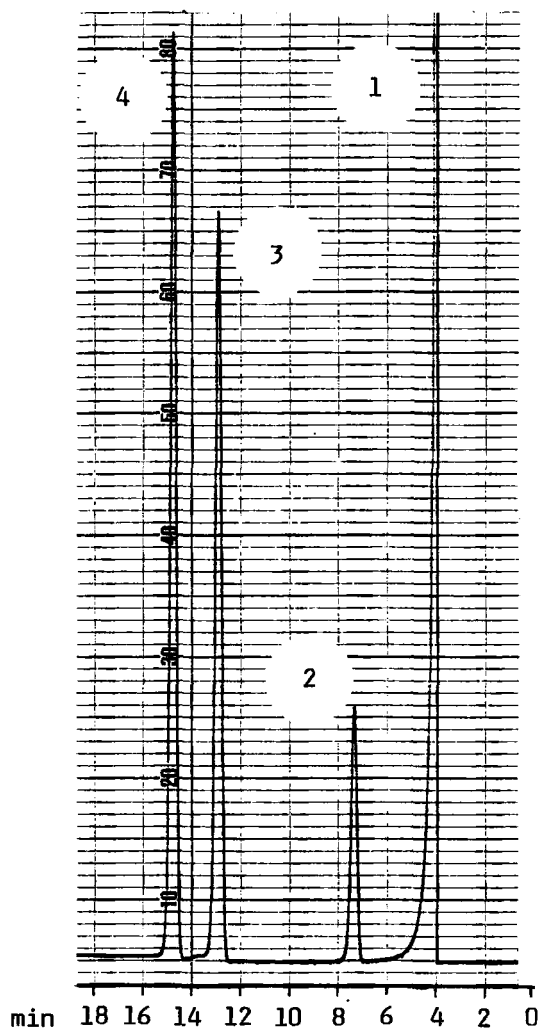
Fig. 8 shows a chromatogram of the drug substance spiked with the degradation product. The flow was set at 2.0 ml/min.

## 7.4 Analysis of the Dosage Forms

### 7.41 Identification

The identification of dibenzepin hydrochloride in the dosage forms can be carried out by thin layer chromatography using silica gel plates with chloroform/cyclohexane/diethylamine 1:8:1 (v/v/v) and subsequent UV visualisation at 254 nm. The most advantageous spraying reagents is Dragendorff's reagent with consecutive spraying by a mixture of 20 ml hydrogen peroxide 30 per cent and 10 ml of ethanol (cf. 7.31).





**Figure 7:** Gaschromatogram of Dibenzepin spiked with the degradation product and octacosane (internal standard).

Instrument: PERKIN-ELMER 900.

Key:

- 1 = dichlormethane (solvent)
- 2 = N-(2-(dimethylamino)ethyl)-N'-methyl-N'-phenyl-  
-1,2-benzenediamine hydrochloride (degradation  
product)
- 3 = dibenzepin
- 4 = octacosane (internal standard)

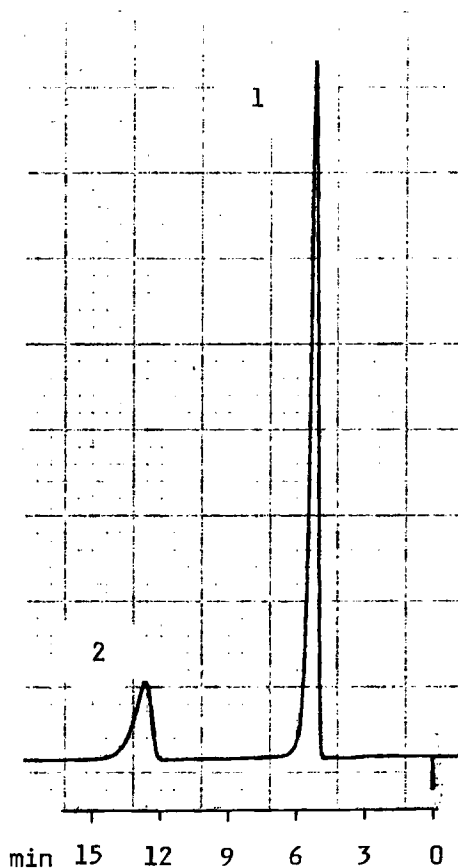


Figure 8: High Performance Liquid Chromatogram of Dibenzepin Hydrochloride spiked with the degradation product.

Reversed-phase Mode, isocratic,  
UV detection at 221 nm.

Key:

- 1 ■ dibenzepin hydrochloride
- 2 ■ N-(2-(dimethylamino)ethyl)-N'-methyl-N'-phenyl-1,2-benzenediamine hydrochloride (degradation product)

Dibenzepin can also be identified by IR spectroscopy after extraction from the dosage form with chloroform.

#### 7.42 Assay

Dibenzepin hydrochloride in Noveril® coated tablets and tablets may be assayed in a non-specific way by direct UV spectrophotometry after extraction with 0.1 N hydrochloric acid or, in case of solutions (injection or concentrate intended for injection by intravenous infusion) after dilution with 0.1 N hydrochloric acid.

A specific assay of dibenzepin hydrochloride in the dosage form may be carried out by tlc followed by UV spectrophotometry (the system can also be used for identification purposes). The active ingredient is extracted with methanol. The chromatographic conditions are: silica gel, mobile phase: chloroform/cyclohexane/diethylamin 1:8:1 (v/v/v). The spot corresponding to dibenzepin is extracted with 0.1 N hydrochloric acid, and the concentration is determined at about 285 nm (shoulder) by spectrophotometry.

A further specific assay is the HPLC determination of dibenzepin hydrochloride after extraction with methanol/water 8:2 (v/v) from the dosage form using LiChrosorb® RP-8 as stationary phase and acetonitrile/1 per cent ammonium carbonate solution 65:35 (v/v) as the mobile phase. UV detection wavelength is set at 221 nm.

#### 7.5 Determination in Body Fluids

The isolation, separation and identification of dibenzepin hydrochloride and its metabolites from urine of man, rabbits and dogs is described in [8]. The methods used are extraction, thin layer and gas chromatography; quantitative determinations were made by UV spectroscopy. Gas chromatographic procedures for the determination of the drug and its basic metabolites via acetylation of the demethylated compounds are described in [9 - 13].

The isolation of dibenzepin hydrochloride from plasma and other body fluids by extraction or column chromatography and its identification by gas chromatography are given in [14,15]. A gas chromatographic determination of the active ingredient and its basic metabolites in biological material after tri-fluoracetylation is described in [16]. In that paper a simple procedure is given for the separation of dibenzepin hydrochloride and its demethylated metabolites by ion-pair extraction.

HPLC may also be used for the separation and determination of the active ingredient [17, 18].

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# DIGOXIN

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## 1. Description

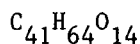
Digoxin is a cardiotonic glycoside obtained from the leaves of Digitalis lanata Ehrhart (Fam. Scrophulariaceae).

### 1.1 Names

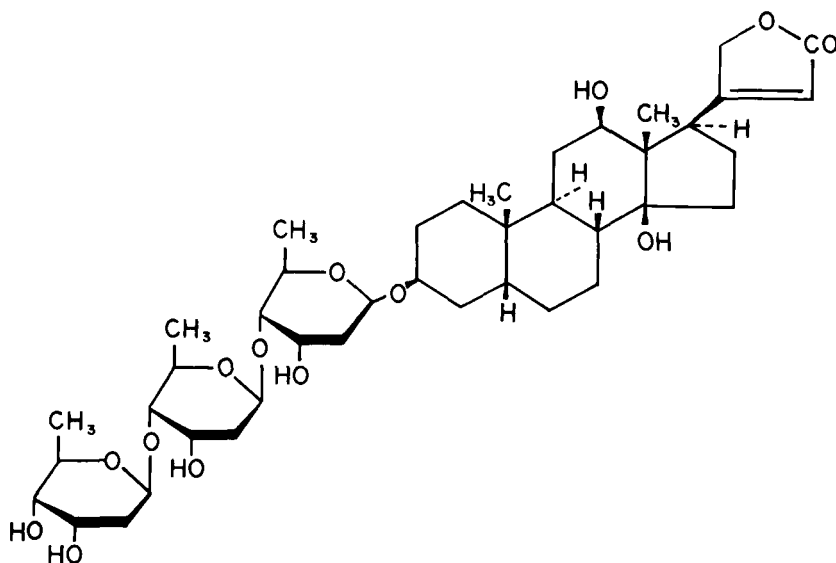
3 $\beta$ -[(O-2,6-Dideoxy- $\beta$ -D-ribo-hexopyranosyl-(1 $\rightarrow$ 4)-O-2,6-dideoxy- $\beta$ -D-ribo-hexopyranosyl-(1 $\rightarrow$ 4)-2,6-dideoxy- $\beta$ -D-ribo-hexopyranosyl)oxy]-12 $\beta$ ,14-dihydroxy-5 $\beta$ -card-20(22)-enolide<sup>2</sup>

Cordioxil, Davoxin, Digacin, Dilanacin, Dixina, Lanocardin, Lanicor, Lanoxin, Rougoxin, Vanoxin<sup>2</sup>

### 1.2 Formula, Structure, Molecular Weight



780.96



### 1.3 Appearance, Color, Odor

Digoxin is an odorless, white crystalline powder.

## 2. Physical Properties

### 2.1 Infrared Spectrum

The infrared spectrum of digoxin is shown in Figure 1.<sup>3</sup> It was taken as a 0.2% dispersion of digoxin in KBr with a Nicolet Model 7199 FT-IR. Table I gives the infrared assignments consistent with the structure of digoxin.<sup>4</sup>

Table I

#### Infrared Spectral Assignments for Digoxin

<u>Band (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3445	O-H stretch
2930	C-H stretch of CH <sub>3</sub> -, -CH <sub>2</sub> -
1725	C=O stretch character- istic of $\alpha$ , $\beta$ unsat- urated $\gamma$ lactone
1625	C=C stretch
1445, 1405, 1375, 1320, 1270	C-H bending vibrations of -CH <sub>3</sub> , and -CH <sub>2</sub> -
1163, 1150, 1080, 1020	C-O stretch for alcohols and ethers
865	C-H bend of trisubsti- tuted C=C

### 2.2 Nuclear Magnetic Resonance (NMR) Spectra

The <sup>1</sup>H and <sup>13</sup>C NMR of digoxin are shown in Figures 2 and 3.<sup>5</sup> Tetramethylsilane is the internal standard in the solvents used for the proton and carbon NMR.

The <sup>1</sup>H NMR was obtained with a Varian XL-100A at 100 MHz with deuterated chloroform as the solvent. The <sup>1</sup>H NMR is very complex and not all protons can be assigned. Protons 18-CH<sub>3</sub> and 19-CH<sub>3</sub>, plus the HOD signal are between 0.82-0.95 ppm. The 4', 4'', and 4''' protons are from 3.16-3.30 ppm. Protons 5', 5'', 5''' appear between 3.70-3.98 ppm, and protons 3', 3'', 3''' and 3 are between 3.98-4.34 ppm. Protons 1', 1'' and 1''' are between 4.80-5.02 ppm.<sup>6</sup>

The <sup>13</sup>C NMR was obtained with a Varian CFT-20 instru-  
ment at 20 MHz. Deuterated dimethylsulfoxide was the  
solvent. Table II gives the carbon assignments for the <sup>13</sup>C  
NMR.<sup>6</sup>



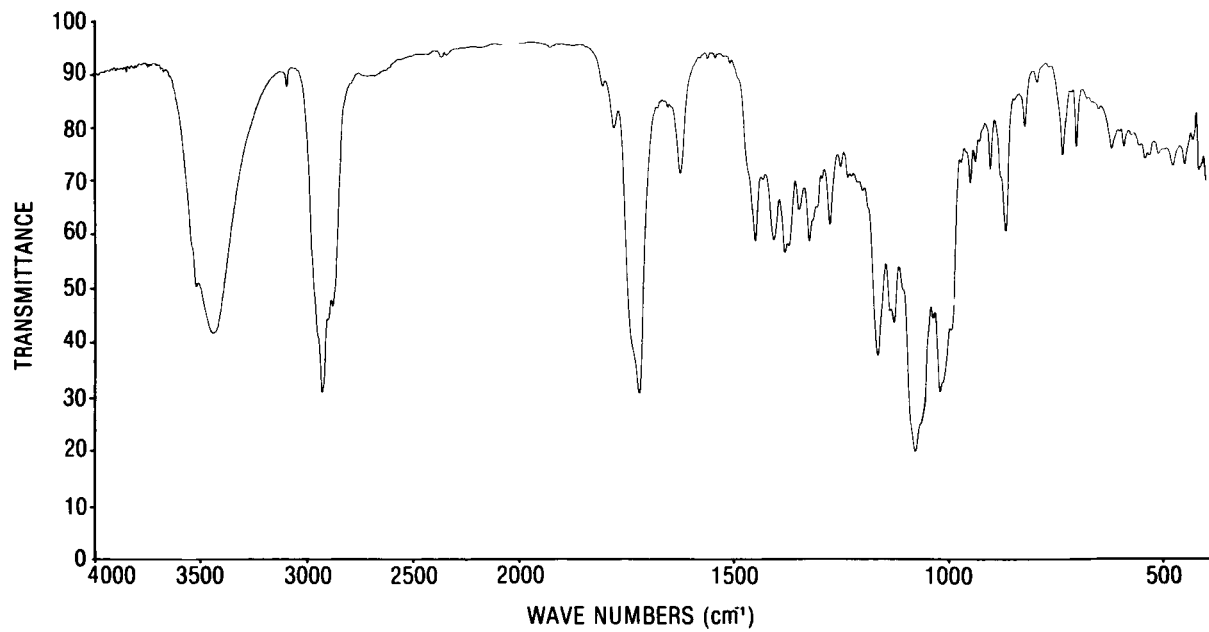


Figure 1 - Infrared Spectrum of Digoxin

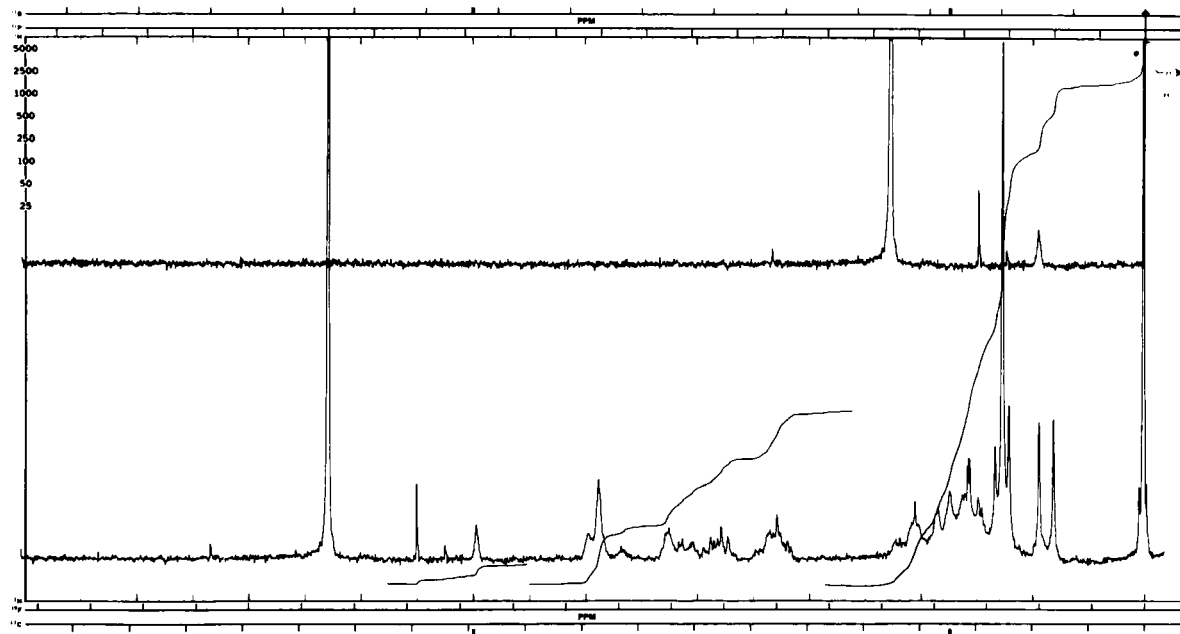


Figure 2 -  $^1\text{H}$  Nuclear Magnetic Resonance Spectrum of Digoxin

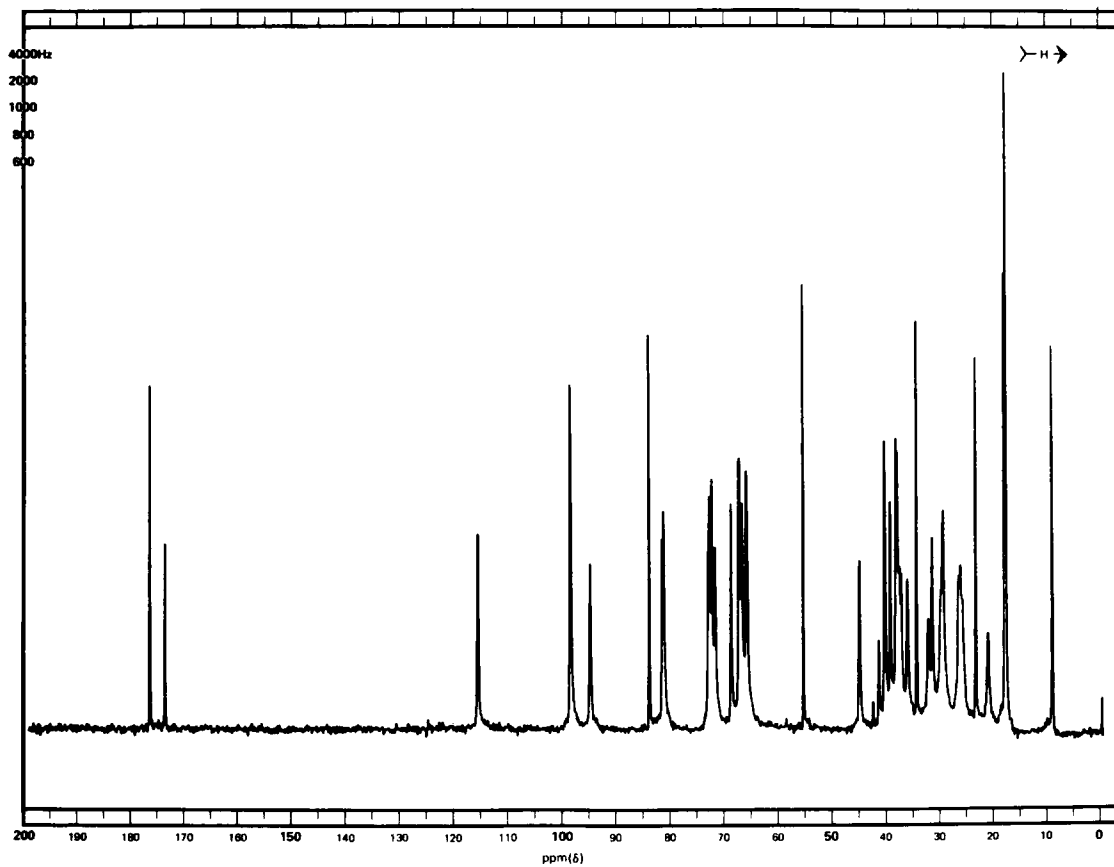
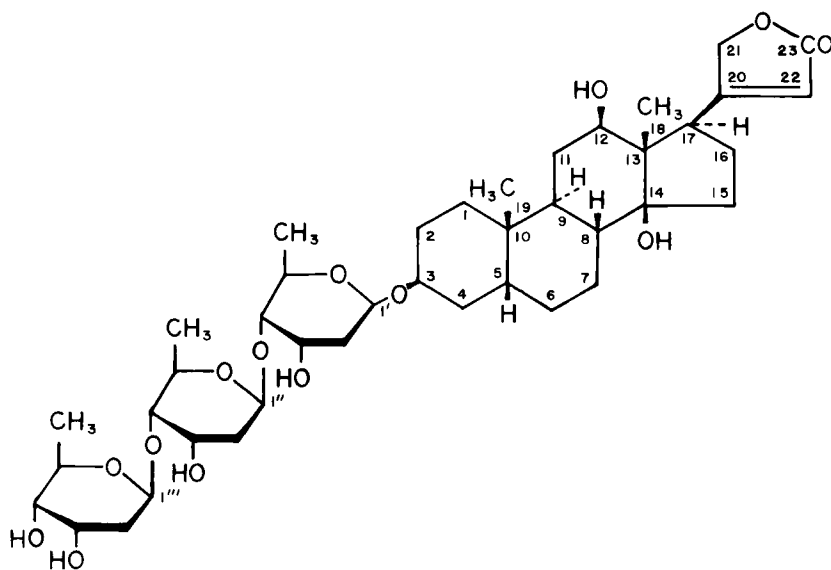


Figure 3 -  $^{13}\text{C}$  Nuclear Magnetic Resonance Spectrum of Digoxin

Table II $^{13}\text{C}$  NMR Assignments for Digoxin

<u>Carbon No.</u>	<u>Chemical Shift (ppm)</u>
7	21.31
10	34.59
13	55.64
14	84.30
17	45.16
18	9.34
19	23.58
20	176.69
22	115.84
23	173.82
1'	98.91
1''	98.91
1'''	95.30

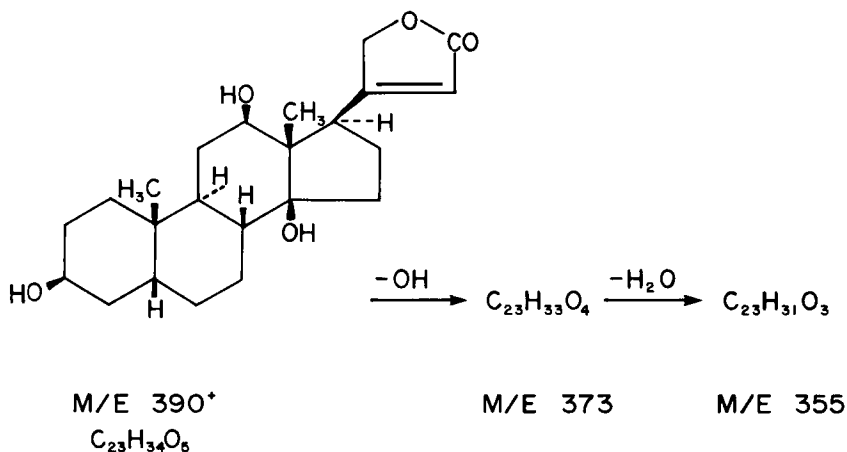


### 2.3 Ultraviolet (UV) Spectrum

The UV spectrum of digoxin in ethanol was taken with a Beckman ACTA CIII UV spectrophotometer and is shown in Figure 4.<sup>4</sup> Digoxin has one maximum in the UV spectrum at 220 nm with  $\epsilon = 1.28 \times 10^4$ .

### 2.4 Mass Spectrum

The mass spectrum of digoxin as shown in Figure 5 was obtained with a Varian MAT CH5-DF mass spectrometer.<sup>7</sup> The direct probe temperature was 290°, and the electron energy was 70 eV. The major fragmentation pattern characteristic of the aglycone portion of digoxin is outlined below.<sup>8</sup>



### 2.5 Optical Rotation

The optical rotation of digoxin has been determined under different conditions.

$$[\alpha]_{\text{Hg}}^{25} + 13.6^\circ \text{ to } 14.2^\circ \text{ (C=10 in pyridine)}^1$$

$$[\alpha]_{\text{D}}^{20} + 18.9^\circ \text{ (C=1 in pyridine)}^2$$

$$[\alpha]_{\text{D}}^{20} + 30.4^\circ \text{ (C=1.2 in alcohol)}^2$$

### 2.6 Melting point

Digoxin melts and decomposes between 230°-265°C.<sup>9</sup>

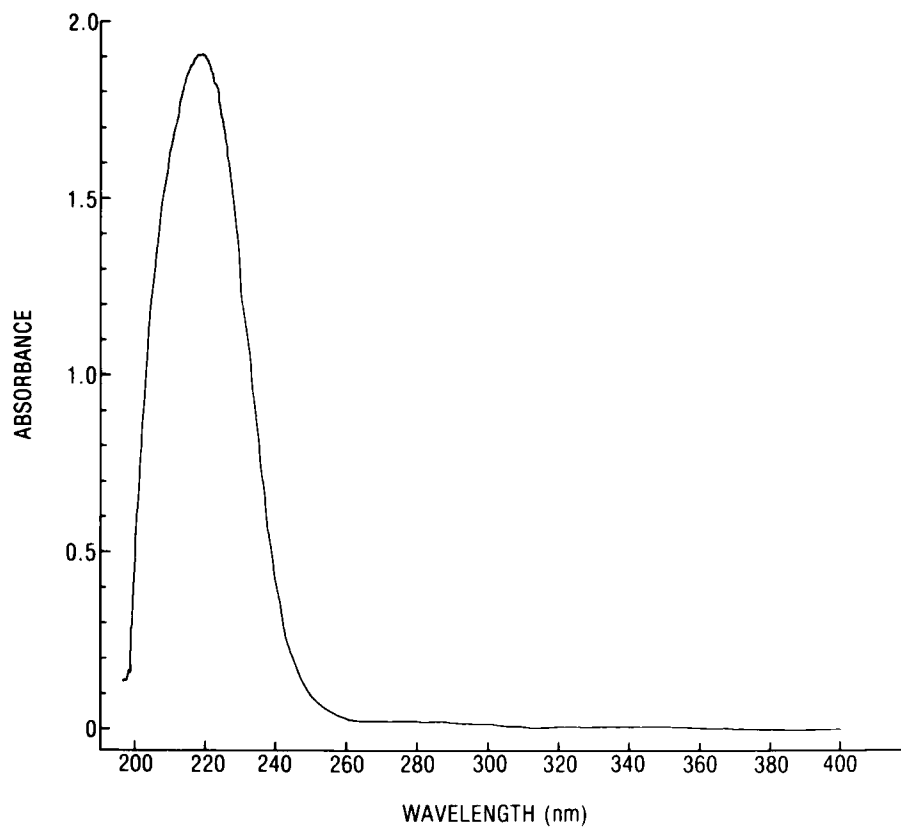


Figure 4 - Ultraviolet Spectrum of Digoxin

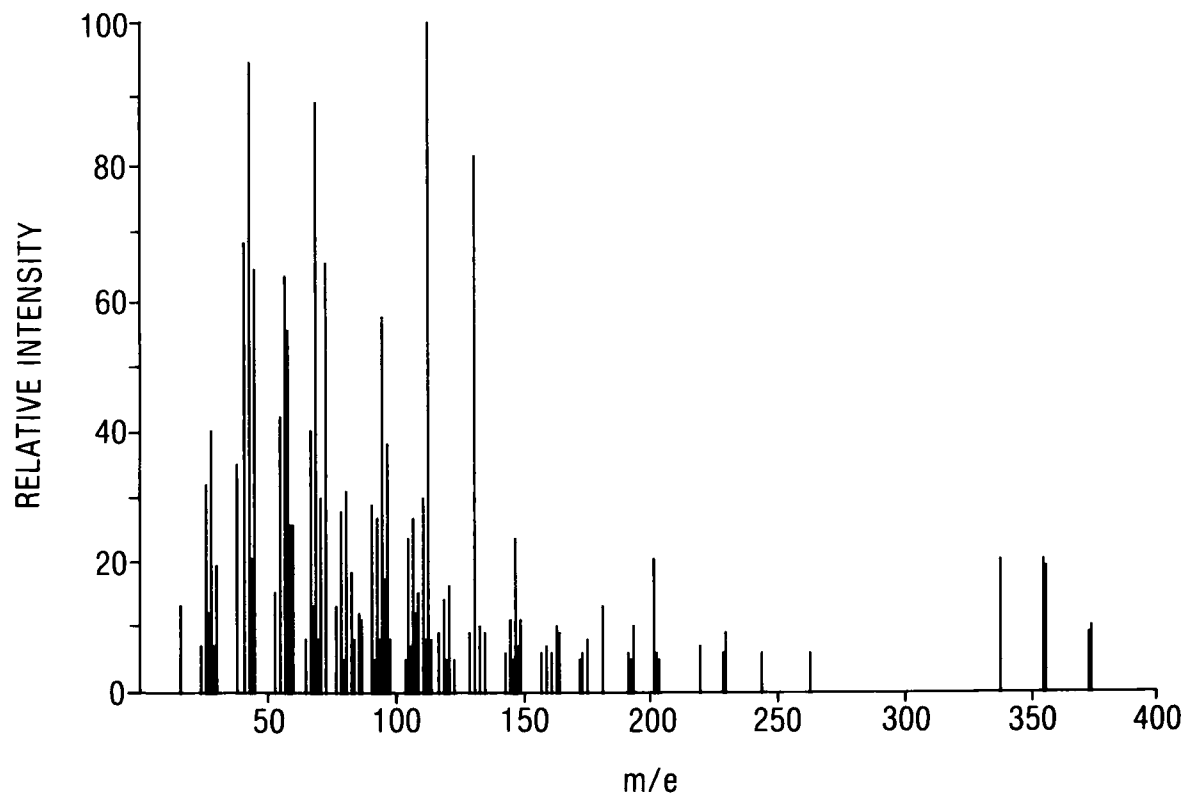


Figure 5 - Mass Spectrum of Digoxin

## 2.7 Solubility

Digoxin is freely soluble in pyridine, slightly soluble in 1:1 ethanol:water, chloroform, and practically insoluble in water and in ether.<sup>1,2</sup>

## 3. Synthesis

No successful synthesis of digoxin has been reported. Digoxin is obtained commercially from the ethanolic extraction of Digitalis lanata leaves followed by chromatographic purification.

## 4. Stability<sup>9</sup>

Digoxin is stable indefinitely when kept in the dark in well closed containers. No degradation is noted in tablets after five years when stored in tightly closed containers.

Solutions of digoxin hydrolyze in the presence of acids yielding digoxigenin bis-digitoxoside, digoxigenin mono-digitoxoside and digoxigenin. The latter degrades further to anhydrodigoxigenin under anhydrous acid conditions.

Neutral solutions of digoxin in ethanol and propylene glycol are stable up to five years.

Digoxin solutions are relatively stable to light except when stored under intense light for long periods of time. Degradation is by apparent opening of the lactone ring and can be detected by a lowering of the ultraviolet absorbance and by HPLC assays.

Only chromatographic procedures can be used to determine digoxin in the presence of all its breakdown products.

## 5. Pharmacokinetics, Metabolism, and Protein Binding

### 5.1 Pharmacokinetics and Metabolism

In man digoxin is 60-80% absorbed and has a biologic half life of 1.5 to 2.0 days.<sup>10</sup> In the anuric patient the half-life is prolonged to four to six days. To determine which dosage form has the best bioavailability digoxin was given by intravenous infusion, intramuscular injection, oral elixir, and tablet to human subjects.<sup>11</sup> The fate of the glycoside is similar regardless of the dosage method used.<sup>12</sup> The bioavailability of the dosage forms was compared by



serum concentration levels and cumulative urinary excretion.<sup>11</sup> The dosage forms, in order of highest to lowest resulting serum concentration levels, and excretion, were intravenous infusion, intramuscular injection, oral elixir, and tablet. An improved digoxin tablet with a more rapid dissolution rate, showed twice the absorption rate of the previous tablet and a forty percent increase in urinary excretion.<sup>13</sup> A new encapsulated liquid digoxin (a soft gelatin capsule containing the glycoside in a dissolved form) was superior in bioavailability to the rapid dissolution tablet and the solution.<sup>14,15</sup> The capsule's absorption approaches that of the intravenous dosage forms.

In postmortem examinations of patients with normal renal function, the highest concentration of digoxin was in the kidney, followed by the heart and liver.<sup>16</sup> The lowest concentration of digoxin was in the brain. Studies in anephric patients and those with renal failure show the highest concentration of digoxin to be in the heart followed by the liver, and the kidney. When digoxin content was measured in samples of left ventricular papillary muscle,<sup>17</sup> skeletal muscle, and plasma of human patients during heart surgery, the papillary muscle digoxin concentration averaged 77 ng/g, the skeletal muscle, 11.3 ng/g and plasma, 1-2 ng/mL. A significant amount of total body digoxin is stored in the skeletal muscle since skeletal muscle represents 43% of the body weight. A relatively wide range of digoxin concentration in atrial heart tissue is commensurate with satisfactory digitalization. Myocardial tissue samples taken two hours after the intravenous administration of tritiated digoxin revealed a significant variation in digoxin concentration in and around the infarcted zone. The infarcted tissue<sup>18</sup> demonstrated a tissue to serum ratio of 12:1. The therapeutic activity of digitalis is likely to depend on the concentration at the active sites in the tissues rather than in the plasma.

The quantity of digoxin excreted each day is a function of the amount present in the body. Excretion during the first twenty-four hours has been determined to be between 20-50% of the dose.<sup>19,20</sup> Digoxin undergoes appreciable biliary excretion after intravenous dosing in man, however, total fecal recovery is low, with figures ranging from 6-20% of the dose of digoxin.<sup>19,10,11</sup> Doherty *et al.*<sup>21</sup> determined that only 6-8% of the given dose of digoxin is recycled through the bowel. Digoxin is excreted predominantly through the kidney.

In dogs, it was found that approximately sixty percent<sup>22</sup>

of the metabolism of digoxin takes place in sites other than the liver. The heart muscle was found to have a negligible role in digoxin metabolism. A significant amount of digoxin is excreted unmetabolized. The following digoxin metabolites are present in the lipid-extractable fraction of urine or plasma : dihydrodigoxin, digoxigenin bis-digitoxoside, digoxigenin mono-digitoxoside, dihydrodigoxigenin monodigitoxide, and digoxigenin.<sup>23</sup> Dihydrodigoxin is the major metabolite. In various animals the activity of dihydrodigoxin has been measured to be 1/7 to 1/20 the activity of digoxin.<sup>24</sup> All glycolytic reduced and nonreduced metabolites were found except for dihydrodigoxigenin<sup>23</sup> which is often detectable only in patients using very high doses of digoxin. Metabolic conversion of digoxin includes the stepwise hydrolysis of the sugar units, conjugation to form water soluble metabolites, epimerization at C-3, and reduction of the lactone ring which destroys the activity of digoxin.<sup>23</sup>

## 5.2 Protein Binding

Digitalis protein binding is important because tissue uptake is related to free drug and not to total drug concentration. The variations reported in protein binding of digoxin may result from differences in methodology and may also occur when using the same method, but in different laboratories.

Significant species differences in binding have been reported for digoxin. Baggot<sup>25</sup> reported the range to be 17-40% binding. Storstein<sup>26</sup> reported the range to be 5-60%. Most investigators found digoxin binding to be about 20%.

Storstein<sup>26</sup> used equilibrium dialysis and ultrafiltration for measuring the protein binding of digoxin. With equilibrium dialysis 21-24% of digoxin was found to be protein bound. The glycoside concentration was within therapeutic range, and the dialysis was performed at room temperature. Serum or human albumin was used for the equilibrium dialysis. Storstein reported that the ultrafiltration results were not accurate.

Doherty and Hall<sup>27</sup> reported that the lack of affinity for serum protein binding for digoxin appears to be a function of its polarity. The polar structure of digoxin tends to render chemical protein binding of the drug less likely to occur.

Protein binding of digoxin was found to be normal in

uremic patients, but decreases during hemodialysis.<sup>26</sup>

## 6. Methods of Analysis

### 6.1 Elemental Analysis

Elemental analysis<sup>2</sup> of digoxin as  $C_{41}H_{64}O_{14}$

C	63.06%
H	8.26%
O	28.69%

### 6.2 Identification Tests<sup>1</sup>

Digoxin is dissolved and diluted with hot ethanol and an aliquot is evaporated to dryness. Acid-ferric chloride TS is added to the residue. A green color develops that slowly changes to a deep green-blue.

Digoxin is dissolved and diluted with hot ethanol. An aliquot of the solution is evaporated to dryness then dissolved in a solution of methanol and chloroform (1:2). The sample is spotted onto Whatman No. 1 filter paper that has been impregnated with a solution of formamide and acetone (3:7). The sample is developed with chloroform saturated with formamide. After development the paper is heated for fifteen minutes at 90°C then sprayed with trichloroacetic acid in chloroform and hydrogen peroxide and reheated to 90°C for ten minutes. The sample is viewed under UV light and compared to the standard.

### 6.3 Fluorometric Analysis

Fluorometry has been used to simultaneously determine digitoxin and digoxin in leaves, tincture, tablets, and drug.<sup>28</sup> An Aminco Bowman spectrofluorometer was used for the determination of the excitation and emission spectra, and a Turner model 110 was the fluorometer used for the analysis. The reagent was a mixture of acetic anhydride, acetyl chloride, and trifluoroacetic acid. Digoxin has two excitation peaks, one at 470 nm, the same as digitoxin, and a second at 350 nm. The fluorescence peaks for both digitoxin and digoxin occur at 500 nm. With a 47B + 2A-12 filter combination the reading was found to be a sum of the fluorescence of digoxin and digitoxin. To correct for digoxin fluorescence the 7-60 + 2A-2ND filter combination was used because it allows the determination of the emission of digoxin alone. The results were linear over the concentration range of 0.5 to 6 µg/mL. The accuracy, based on 2 µg/mL was 99.2% of theory.

Fluorimetric analysis was also used for the determination of digoxin in tablets.<sup>29</sup> A Technicon automatic analyzer was used for the analysis. The reagents and solutions were, 70% SD3A alcohol in water, hydrochloric acid, ascorbic acid, hydrogen peroxide, and standard digoxin. Three standards of appropriate levels and samples of the intact or powdered tablets were used. Excitation and emission wavelength maxima for digoxin were 350 nm and 490 nm respectively. Spectral measurements were made on a Farrand Spectrofluorometer. The procedure was stability indicating, and a linear relationship existed between fluorescent intensity and digoxin concentration. The relative standard deviation of a 0.25 mg digoxin sample was  $\pm 1.2\%$ . None of the tablet excipients interfered with the procedure.

The following fluorimetric assay procedure has also been used for the analysis of digoxin in tablets.<sup>30</sup> Ten mL of 80% alcohol was added to a tablet, in a volumetric flask, warmed on a steam bath until the tablet was dispersed, and the alcohol boiled. The mixture was cooled, swirled, and diluted to 20 mL with 80% alcohol. After standing for 15 minutes 5 mL of the supernatant was pipetted into a 20 mL volumetric flask and diluted to volume with 80% alcohol. Three mL of 0.1% solution of ascorbic acid in methanol, 0.2 mL of .009 M hydrogen peroxide in water were added to a 1 mL aliquot of the sample solution. The solution was diluted to ten mL with hydrochloric acid and allowed to stand for two hours in the dark. The standard was prepared in a similar manner. For the fluorescence measurement the excitation maximum was at 355 nm and the emission maximum was at 490 nm.

## 6.4 Chromatography

### 6.41 Paper Chromatography

Paper chromatography<sup>31</sup> has been used to separate the components of a digitalis tincture. Whatman 3MM paper impregnated with formamide and developed in chloroform gave an  $R_f = 0.33$  for digoxin. a variety of spray reagents were used<sup>f</sup> to detect digoxin.

### 6.42 Thin Layer Chromatography

Table III gives various thin layer chromatography systems which have been used for the separation of digoxin.

Table III  
Thin Layer Chromatography for Digoxin

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, R<sub>f</sub> or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel G	Cyclohexane-acetone- acetic acid (49:49:2)	50% aq sulfuric acid	Digoxin appears as a blue spot under 385 nm UV light	32
	or Ethyl acetate-water- methanol (80:5:5)	or 30% aq soln chloramine and 25% alcoholic soln trichloroacetic acid (1:4)		
Silica Gel F	Ethyl acetate-methanol- water (80:5:5)	6 g of trichloroacetic acid in 25 mL chloro- form and 0.5 mL 30% w/v hydrogen peroxide	Digoxin 0.3	33
Silica Gel (non-fluorescent)	Chloroform-acetone (1:1)	50% methanolic sulfuric acid	Digoxin, digoxigenin bis-digitoxoside, digoxigenin mono- digitoxoside, $\alpha$ -anhydrodigoxigenin, $\beta$ -anhydrodigoxigenin Comment: 2-25 hrs continuous development	34
	Dichloromethane- methanol (9:1)		Digoxin, gitoxigenin, digitoxigenin	

Table III (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, R<sub>f</sub> or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel 60 F <sub>254</sub>	Ethylacetate-dichloro- methane-methanol-water (60:36:3.5:2)	20% v/v orthophosphoric acid	Gitoxigenin, digoxigenin, β-acetyl digoxin, digoxigenin mono-digitoxoside, α-acetyl digoxin, digoxigenin bis-digi- toxoside, gitoxin, digoxin, digitoxin, digitoxigenin	35
	Chloroform-pyridine (60:10)		Digitoxin, digoxin	
	Dichloromethane- methanol (90:10)		Digitoxin, digoxin	
Silica Gel F <sub>254</sub>	Chloroform-acetone (1:1)	20% v/v orthophosphoric acid	Digoxigenin, dig- oxigenin mono- digitoxoside, digoxigenin- bis-digitoxoside, digoxin	36
Kieselgel 60 DC-Fertigplatten	Ethylacetate-dichloro- methane-methanol- water (120:72:7:4)		All cardenolides are referenced relative to digoxin.	37

Table III (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, R<sub>f</sub> or Relative Order of Elution</u>	<u>Ref.</u>
	and Dichloromethane- methanol (9:1)		<u>Comment:</u> Mobile phase 1: continuous development 3 hrs, mobile phase 2: continuous development 2 hrs. The plate is turned 90° after development by mobile phase 1.	

### 6.43 Gas Chromatography

Digoxin, as a tablet or as the powdered drug,<sup>32</sup> was converted to digoxigenin for analysis by gas chromatography. The separation was achieved by using three columns, (A) a two meter glass U tube packed with 2.5% OV-1 on 80-100 mesh Chromosorb A, (B) a 0.5 meter copper U tube with 2.5% OV-1 on 80-100 mesh Chromosorb A, and (C) 3% OV-17 on 80-100 mesh Chromosorb A. Cholesterol was used as the internal standard. The oven temperature was 285°C. The injection port and the flame ionization detector temperature were 330°C. All injections were made with a 5  $\mu$ L syringe. The detection range was 0.05-0.2 mg. There was little difference in the retention times of the silylated drug and standard versus those of the unsilylated drug and standard.

	<u>Retention times (min)</u>		
	<u>Column A</u>	<u>B</u>	<u>C</u>
Cholesterol unsilylated	2.0	0.67	0.5
silylated	2.0	0.67	0.5
Digoxigenin unsilylated	15.0	5.33	3.67
silylated	15.0	5.0	3.67

### 6.44 High Performance Liquid Chromatography

Table IV gives various HPLC systems used for digoxin.

### 6.5 Polarography

Polarography has been used for the assay of digoxin tablets.<sup>47</sup> The working electrode was a dropping mercury electrode with a one second drop time, and the reference electrode was a saturated calomel electrode (SCE) with a platinum wire as an auxiliary electrode. The linear potential sweeps were constant at 5 mV/sec, and the pulse modulation was 25 mV. A 2-mL aliquot of the extraction of the ground tablets plus 0.2 mL of 0.2 M TBAI, tetrabutylammonium iodate, or of 0.2 M TBAH, tetrabutylammonium hydroxide, the supporting electrolytes, was added to 2 mL of isopropanol. Before each experiment the solution was deaerated with isopropanol saturated nitrogen, which was also passed over the solution during the assay. The potential was scanned cathodically from -1.8 volt. The peak potential of digoxin was -2.285<sub>-4</sub> volts. The useful analytical range of the assay was  $5 \times 10^{-4}$  M to  $2.5 \times 10^{-6}$  M of digoxin.



Table IV

HPLC Systems for Digoxin

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (mL/min) or Pressure</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref.</u>
Li Chrosorb SI60 (25 cm x 3 mm id)	n-Butanol- acetonitrile-heptane- water (230:100:700:10)	1.3	3.5	225 nm	38
	t-Butanol-acetonitrile- heptane-water (220:70:800:10)	2.2	10		
	(204:93:712:10.4)		3.6		
	n-Pentanol-aceto- nitrile-iso-octane- water (270:93:660:9.3)	1.3	3.8		
	(230:100:700:10)	1.4	5.2		
	(170:60:620:10)	1.3	10.4		
	(175:60:620:6)		8.2		

Table IV continued

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (mL/min) or Pressure</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref.</u>
Merckosorb SI60 5 $\mu$ m (15 cm x 3 mm id)	Comment: The digitalis glycosides are derivatized with 4-nitrobenzoyl chloride (4N BCl)			254 nm or 260 nm	39
	n-Hexane-methylene chloride-acetonitrile (10:3:3)	1.5	5.6		
	n-Hexane-chloroform- acetonitrile (30:10:9)	1.5	5.9		
Li Chrosorb SI60 5 $\mu$ m (15 cm x 3 mm id)	8% Methanol in methy- lene chloride saturated with water	2.0	1.3	230	40
Nucleosil C <sub>18</sub> (30 cm x 3.5 mm id)	37% Acetonitrile in water	1.4	4	220	40
	40% Solution of 1:1 acetonitrile- dioxane in water	1.3	5.4		

Table IV continued

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (mL/min) or Pressure</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref.</u>
Whatman ODS-1 (25 cm x 4.2 mm id)	540 mL of acetonitrile diluted to two liters with water	3.0	5.6 (tablets)	220 nm	41
			7.0 (injection)		
			5.4 (pediatric injection)		
	450 mL of acetonitrile diluted to two liters with water	3.0	12.4 (elixir)		
Li Chrosorb SI60 (25 cm x 4 mm id)	Cyclohexane-absolute ethanol-acetic acid (60:9:1)	2	8	265,234 nm	42
Whatman ODS-1 (30 cm x 4.2 mm id)	780 mL Acetonitrile diluted to three liters with water	2.25	9	220 nm	43
Perisorb RP (1 mm x 2 mm id)	Water with 25% aceto- nitrile	0.75	4.5	254 nm	44

Table IV continued

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow (mL/min) or Pressure</u>	<u>Retention Time (min)</u>	<u>Detection</u>	<u>Ref.</u>
Zorbax-SIL (25 cm x 2.1 mm id)	6% Methanol + 0.15% acetic acid in methylene chloride	1500 psi	4.5	254 nm	45
	3% Methanol + 0.1% acetic acid in methylene chloride	0.5 or 1200 psi	10	254 nm, 235 nm	46

## 6.6 Colorimetry

An alkaline dinitrobenzene<sup>48</sup> reagent has been used for a colorimetric assay of crystalline powder, tablets, injections, and elixirs containing digoxin. The dinitrobenzene reagent was added to standard and sample preparations that have been evaporated to dryness. The mixture was allowed to stand for five minutes, with frequent stirring, at a room temperature not exceeding 30°C. The absorbance of the resulting blue color was measured at 620 nm versus the reagent blank and the USP digoxin standard.

The following method of assaying dosage samples used a color reagent of glacial acetic acid<sup>49</sup> containing ferric chloride and sulfuric acid. After an initial extraction procedure tailored to the sample type, the sample was dissolved or diluted in a chloroform-methanol solution (65:35) then diluted with glacial acetic acid. An aliquot of digoxin solution was diluted with color reagent and allowed to stand for two hours. The absorbance of the sample was measured at 590 nm versus that of a digoxin standard.

The following two colorimetric procedures have been used for the assay of tablet samples. The first employed an alkaline sodium picrate reagent.<sup>50</sup> A crushed digoxin tablet was placed in a 10-mL volumetric flask and diluted with 6-mL of absolute alcohol. The flask was heated to 40°C and shaken for two hours. The solution was diluted to volume with alcohol, then centrifuged. A 3-mL aliquot of the reagent was added to a 5-mL aliquot of the sample. The solution was stored in darkness for 30 min. The absorbance was measured at 490 nm versus a reagent blank.

The Xanthyrol<sup>50</sup> method was another tablet assay procedure. In a 50-mL volumetric flask a tablet was crushed in a solution of three mL of hot chloroform/methanol (65:35) and two mL of glacial acetic acid. Twenty mL of xanthyrol reagent was added to the mixture. The flask was heated for five min in a 75°C water bath then cooled for five min in an ice bath. The standard and blank were prepared in a similar manner. The absorbance was measured at 540 nm.

## 7. Methods of Analysis - Biochemical Applications

### 7.1 Chromatography

#### 7.11 Paper Chromatography

Table V gives various paper chromatography systems

Table V

Paper Chromatography for Digoxin and Metabolites

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf, or Relative Order of Elution</u>	<u>Ref.</u>
Whatman No. 1 filter paper impregnated with formamide (30% in acetone)	Chloroform saturated with formamide	25% trichloroacetic acid solution in chloroform with four drops of hydrogen peroxide/50 mL	dihydrodigoxin,	51
Whatman No. 3 filter paper soaked with formamide-acetone (1:3)	Chloroform-methanol (1:1)	m-dinitrobenzene	digoxin 0.50	52

which have been used for the separation of digoxin and its metabolites.

### 7.12 High Performance Liquid Chromatography

Digoxin and its metabolites have been separated and assayed by reverse phase high performance liquid chromatography.<sup>53</sup> The column was a  $\mu$ Bondapak C<sub>18</sub> (30 cm x 4 mm id). The sample solvent was 95% ethanol, and the injection size was 50-75  $\mu$ L. The detector was set to 220 nm. Listed below are four mobile phases that achieved the desired separation.

#### Isocratic systems:

The flow rate was 3 mL/min

1. 25% acetonitrile in water  $R_t$  of digoxin was 13 min.
2. 33% acetonitrile in water  $R_t$  of digoxin was 23 min.

#### Gradient systems:

The flow rate was 2.2 mL/min

1. 25% acetonitrile in water to 40% acetonitrile in water at 5%/min.  $R_t$  of digoxin was 10 min.
2. 100% water to 30% acetonitrile in water at 6.67%/min.  $R_t$  of digoxin was 23 min.

### 7.13 Column Chromatography

Column chromatography<sup>54</sup> has been used for the separation of digoxin and dihydrodigoxin extracted from urine samples. The adsorbent was diethylaminoethoxypropylated Sephadex LH-20 (DEAE-Sephadex LH-20). The mobile phase was chloroform-methanol (85:15). Samples were applied in 0.2-0.5 mL volumes of eluting solvent. The flow rate for a 40 x 1.0 cm column was 0.25 mL/min.

Dihydrodigoxin  $V_e/V_t = 0.25$

Digoxin  $V_e/V_t = 0.34$

The flow rate for a 36 x 2.5 cm column was 0.20 mL/min.

Dihydrodigoxin  $V_e/V_t = 0.43$

Digoxin  $V_e/V_t = 0.48$

### 7.14 Thin Layer Chromatography

Table VI gives various thin layer chromatography systems which have been used for the separation of digoxin and its metabolites extracted from biological samples.

### 7.15 Gas Chromatography

A single column gas chromatographic determination<sup>62</sup> of digoxin and its metabolites has been achieved with either isothermal or temperature programming. Digoxin and its metabolites were converted to trimethylsilyl (TMS) derivatives prior to analysis. The column (U shaped, 1 ft x 4 mm id) was packed with 1.6% SE 30 on 80-100 mesh Gas Chrom Q. The sample injection volume was 10  $\mu$ L. The instrument used was a Barber-Colman 5000 gas chromatograph equipped with a hydrogen flame ionization detector. Under isothermal conditions the column temperature was set to 300°C and the detector temperature was set to 320°C. The injection block temperature was maintained at column temperature. The nitrogen flow was 125 mL/min. The retention time of digoxin was approximately eighteen minutes. With temperature programming from 230°C to 330°C at 6°C/min, one minute initial delay, the retention time of digoxin was approximately twenty-four minutes. The detector temperature was 340°C and the nitrogen flow was 60 mL/min.

Digoxin and its metabolites, derivatized with heptafluorobutyric anhydride,<sup>60,56</sup> have been resolved on a gas chromatographic column packed with 3% OV-1 on Gas Chrom Q. Digoxin and its metabolites were extracted from urine, plasma, biological tissue, and fecal samples. The compounds were initially separated by paper and/or thin layer chromatography. Before the extraction <sup>3</sup>H-digoxin can be added as an internal standard. The gas chromatograph used was a Tracer MT-220 with a <sup>63</sup>Ni electron capture detector. With a U-shaped column (4 ft x 2 mm id) at 250°C and a detector at 350°C the retention time for digoxigenin HFB was nine minutes. With a U-shaped column (6 ft x 2 mm id) at 250°C and detector at 325°C the digoxigenin HFB retention time was eight minutes.

A Varian CH-7 GC-MS combination<sup>56</sup> was used for an determination of digoxin and its metabolites. For the GC a column packed with 3% OV-1 on Chromosorb W AW DMCS 100/120 (6 ft x 2 mm id) was used. The column temperature was 250°C, injector temperature, 260°C, and the molecular separator, 260°C. For the mass spectrometer, electron energy was 20 eV, the ion source temperature, 250°C, and the trap current, 300  $\mu$ A. The accelerating voltage was 3KV and the



Table VI

Thin Layer Chromatography for Digoxin

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel G	Cyclohexane-acetone- acetic acid (65:33:2)	Lieberman-Burchard (acetic anhydride- sulfuric acid-ethanol (5:5:50))	Digoxin: 0.21 <u>Comment:</u> Plates are developed six times to a height of 15 cm.	55
	Chloroform-ethanol (2:1)		Digoxin: 0.77 <u>Comment:</u> Plates are developed once to 10 cm	
Silica Gel GF	Chloroform-acetone (13:7)		Digoxin: 0.32	56
Silica Gel H	Cyclohexane-acetone- acetic acid (65:33:2)	20% sulfuric acid soln or Anisaldehyde reagent (0.5 mL anisaldehyde, 1.0 mL sulfuric acid, 50 mL acetic acid)	Digoxin: 0.09 <u>Comment:</u> plates developed four times	57

Table VI (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel F <sub>254</sub>		Solution of conc sulfuric acid in ethanol (1:4)		58
	Cyclohexane-acetone- acetic acid		Digoxin: 0.16 (lined tank)	
	(49:49:2)		0.33	
	(49:49:2)		0.34 (lined tank)	
	(45:45:10)		0.59	
	(16:80:4)		0.13	
	Chloroform-pyridine (64:6)			
% formamide in acetone for impregnation				
10%	(64:6)		0.38	
	2-Butanone-xylene-formamide		0.12	
10	(50:50:0)			
10	(50:50:4)		0.09	
15	(50:50:4)		0.10	
20	(50:50:4)		0.09	
10	(70:30:0)		0.36	
	2-Butanone-xylene			
15	(50:50)		0.16	

Table VI (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel G or G <sub>254</sub>	Ethyl acetate-chloro- form-acetic acid (90:5:5)		Digoxin 0.15 one development 0.28 two developments	59
	Cyclohexane-acetone- acetic acid (49:49:2)		Digoxin: 0.29	
	Cyclohexane-acetone- acetic acid (65:33:2 and Cyclohexane-acetone- acetic acid (49:49:2)		0.36 one development in each mobile phase	
	Cyclohexane-acetone-acetic acid (65:33:2) and Ethyl acetate-chloroform (9:1)		0.12 one development in each mobile phase	
	Chloroform-isopropanol-acetone (80:5:15)		0.18 two developments	

Table VI (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf or Relative Order of Elution</u>	<u>Ref.</u>
Silica Gel F <sub>254</sub>	Chloroform-methanol (saturated with AgNO <sub>3</sub> )-ammonia (9:1:1)	Chose one of following: (1) 25% trichloroacetic acid soln in chloroform with four drops of hydrogen peroxide per 50 mL. (2) acetic anhydride- sulfuric acid-abs ethanol (5:5:100)  (3) 0.05 mL p-anisaldehyde, 0.2 mL conc sulfuric acid, 10 mL acetic acid  (4) 20 mg ascorbic acid, 19 mL methanol, 30 mL conc hydro- chloric acid, 2.1 µL 30% hydrogen-peroxide.  (5) 10 mL of 3% aq soln chloramine T, 40 mL 25% trichloroacetic acid in ethanol	0.33 two developments	51

Table VI (continued)

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>Spray Reagent</u>	<u>Comment, Rf or Relative Order of Elution</u>	<u>Ref.</u>
Cellulose (MN-300) predipped with formamide in acetone	Chloroform saturated with formamide	Reagent 5 above	0.33	51
Mallinckrodt Chromar 7GF	Isopropyl ether- methanol (9:1)		0.09 developed five times	
	Isopropyl ether- methanol (9:1) and 2-Butanone-chloroform (3:1)		0.26 developed four times in mobile phase 1, developed one time in mobile phase 2	
Kieselgel 60 DC Fertigplatten	Chloroform-methanol- acetone-water (64:6:28:2)		Digoxin: 0.24	61

Any of the following  
techniques can be used for  
enhanced detection:  
(1) Chloramine-trichloro-  
acetic acid spray  
(2) HCl vapor  
(3) Coating the plate with  
a thin film of parafin

spectrum was scanned every six seconds.

## 7.2 Polarography

The polarographic analysis<sup>63</sup> of digoxin has been used for assaying the drug as well as blood samples containing the drug. The study of the polarographic characteristics of digoxin in a 50% alcohol solution containing tetraethyl ammonium hydroxide showed a half wave potential of -1.965 volts for an alcoholic solution of the drug and -1.958 volts for the drug extracted from blood samples. At concentrations of 0.1-0.4  $\mu\text{g}$  of digoxin in the blood, the error of the method was  $\pm 0.02 \mu\text{g}$ .

## 7.3 Radioimmunoassay

Employing  $^3\text{H}$  digoxin tracer and antiserum solutions available in a commercial kit, optimum conditions were determined for the radioimmunoassay of digoxin in plasma, serum, and urine.<sup>64</sup> A summary of the procedure is given below.

Phosphate buffered saline solution, plasma, and 30% ethanol water were added to each tube and vortexed. The antiserum was added, vortexed, and preincubated, then the tracer solution was added, vortexed, and incubated. The charcoal suspension was added, vortexed, and centrifuged. The supernatant was decanted into 15 mL of liquid scintillation fluid and counted. The range of the assay was 0.05  $\mu\text{g/mL}$  to 5  $\text{ng/mL}$  of digoxin.

Tritiated digoxin has also been used for the determination of digoxin in liver tissue.<sup>65</sup> A liquid-liquid extraction was used to obtain the glycoside. The radioactivity was determined with a liquid scintillation counter. The solvent was 5 mL of 95% ethanol plus 15 mL of toluene. The total counting volume contained 4 g/L of 2,5 diphenyloxazole (PPO) and 50 mg/L of 1,4-bis-2(5-phenyloxazole)-benzene (POPOP). The average recovery rate for the procedure was 95.6% and the sample size was 1 mg.

The amount of digoxin in human plasma has been assayed by radioimmunoassay with an iodinated tracer.<sup>66</sup> The reagents for the assay were digoxin, labelled digoxin (3-O-succinyl digoxigenin [ $^{125}\text{I}$ ]tyrosine derivative), a dilute phosphate buffer containing sodium chloride, bovine albumin powder, sodium azide, anti-digoxin serum, dextran coated charcoal, and normal digoxin free human serum. All standards and specimens were set up in assay tubes and had cold dextran-

coated charcoal suspension added. The tubes were centrifuged and the supernatant placed in a separate assay tube. The supernatant fluid and charcoal were both counted for one minute. Digoxin values of  $\mu\text{g/L}$  of plasma were calculated from a standard curve of the percent tracer bound versus  $\mu\text{g}$  of digoxin per liter. The useful working range of the assay is 0.2 to 8  $\mu\text{g/L}$  of plasma. Fifty  $\mu\text{L}$  of plasma was used.

The amount of  $^{125}\text{I}$ -labelled digoxin has been determined in a 10- $\mu\text{L}$  sample of serum with a modification of a Curtis Digoxin R/A kit assay procedure. The following is a description of the micro-radioimmunoassay procedure. Each polymer tablet was dissolved in the sodium chloride solution, and 100- $\mu\text{L}$  aliquots of the solution were pipetted into the test tubes. Ten  $\mu\text{L}$  aliquots of each standard (0-4.8  $\mu\text{L}$  of digoxin/liter) and patients sera were pipetted into a polymer slurry, mixed, and let stand for ten minutes. Ten  $\mu\text{L}$  of  $^{125}\text{I}$ -labelled digoxin was pipetted into each test tube, mixed and let stand for 30 min. Twice, saline was added to each test tube, centrifuged, and decanted. The contents of the test tubes were counted for ten minutes for radioactivity.

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# DOXORUBICIN

*Aristide Vigevani and Martin J. Williamson*

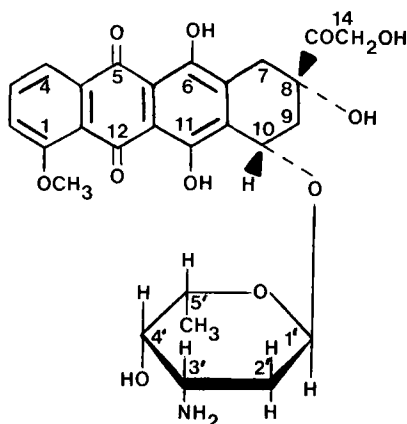
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1. Description1.1 History

Doxorubicin is an antineoplastic antibiotic isolated from a culture of Streptomyces peucetius var. caesius or by chemical synthesis from daunorubicin. The injectable dosage form is supplied as the hydrochloride salt in combination with lactose as a freeze-dried powder.

1.2 Name, Formula, Molecular Weight

Doxorubicin is chemically named (8S-10S)-10-(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxo-hexopyranosyl)oxy-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-hydroxyacetyl-1-methoxy-5,12-naphthacenedione. (CAS-23214-92-8). Originally named (7S:9S)-9-hydroxyacetyl-4-methoxy-7,8,9,10-tetrahydro-6,7,9,11-tetrahydroxy-7-O-(2,3,6-trideoxy-3-amino- $\alpha$ -L-lyxo-hexopyranosyl)-5,12-naphthacenedione.


 $C_{27}H_{29}NO_{11}$ 

MW 543.5

Hydrochloride salt (CAS-25316-40-9)

 $C_{27}H_{29}NO_{11} \cdot HCl$ 

MW 580.0

### 1.3 Appearance, Color

The hydrochloride salt is a red, free-flowing crystalline powder, and the freeze-dried formulation containing lactose is a red cake.

## 2 Physical Properties

### 2.1 Infrared Spectrum

A review of the carbonyl absorptions of antineoplastic (antitumor) anthracyclines has been published<sup>1</sup>. The infrared spectrum of doxorubicin hydrochloride recorded from a KBr pellet (0.4)% on a Perkin-Elmer model 457 grating spectrophotometer is shown in Figure 1. The interpretation of the main absorption bands is given in Table 1.

TABLE 1

Infrared spectrum of doxorubicin hydrochloride

IR Absorption Band, $\text{cm}^{-1}$	Assignments
3560-3160 3160-2300	O-H stretch (hydrogen bonded) $\text{NH}_3^+$ stretch and OH stretch (hydrogen bonded)
1724	C=O (ketone)
1613 and 1580	C=O stretch (intra hydrogen bonded quinone)
1282	C-O-C stretch (ether)
1115	C-O (tertiary alcohol)
1071	C-O (secondary alcohol)
1008	C-O (primary alcohol)

### 2.2 Nuclear Magnetic Resonance Spectra

Proton magnetic resonance spectrometry has been extensively used as a fundamental tool for the determination of the structure of daunorubicin and related compounds<sup>2,3,4</sup>. The  $^1\text{H}$ -NMR spectrum of adriamycinone pentaacetate in  $\text{CDCl}_3$  has been described and tentatively assigned<sup>5</sup>. The  $^1\text{H}$ -NMR spectrum of doxorubicin hydrochloride in  $\text{DMSO}-d_6$  solution recorded at 100 MHz on a Varian HA-100 spectrometer at 80°C (for better resolution) is shown in Figure 2. The interpretation of the spectrum is given in Table 2.

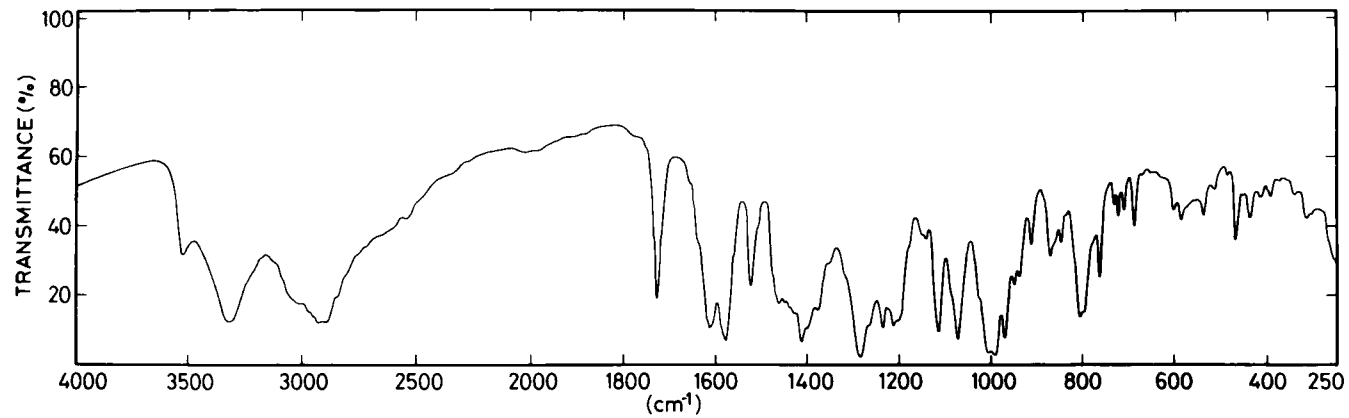


Figure 1. Infrared Spectrum of Doxorubicin

TABLE 2

<sup>1</sup>H-NMR data of doxorubicin hydrochloride in DMSO-d<sub>6</sub> solution at 80°C (TMS as internal reference).

Proton	Multiplicity <sup>a</sup>	δ (ppm)	J or W <sub>H</sub> (Hz)
CH <sub>3</sub> -5'	d	1.15	6.5
H <sub>2</sub> -2'	m	1.77	-
H <sub>2</sub> -9	m	2.15	-
H <sub>2</sub> -7	s	2.92	-
H-3'	m	3.31	-
H-4'	bs	3.62	6.0
CH <sub>3</sub> O	s	3.94	-
H-5'	dq	4.14	6.5 and 1.0
H <sub>2</sub> -14	s	4.57	-
OH-8 OH-4' }	bs	4.46	-
H-10	bs	4.90	10.0
H-1'	bs	5.25	7.0
H-3	t	7.53	7.0
H-4 H-2 }	d	7.79	7.0
NH <sub>3</sub> <sup>+</sup>	bs	7.96	-
OH-6 OH-11 }	two s	13.08 <sup>b</sup> and 13.85 <sup>b</sup>	

a) s - Singlet; d = Doublet; t = Triplet;  
m = Multiplet; bs = Broad signal; dq = Double  
quartet.

b) At room temperature.

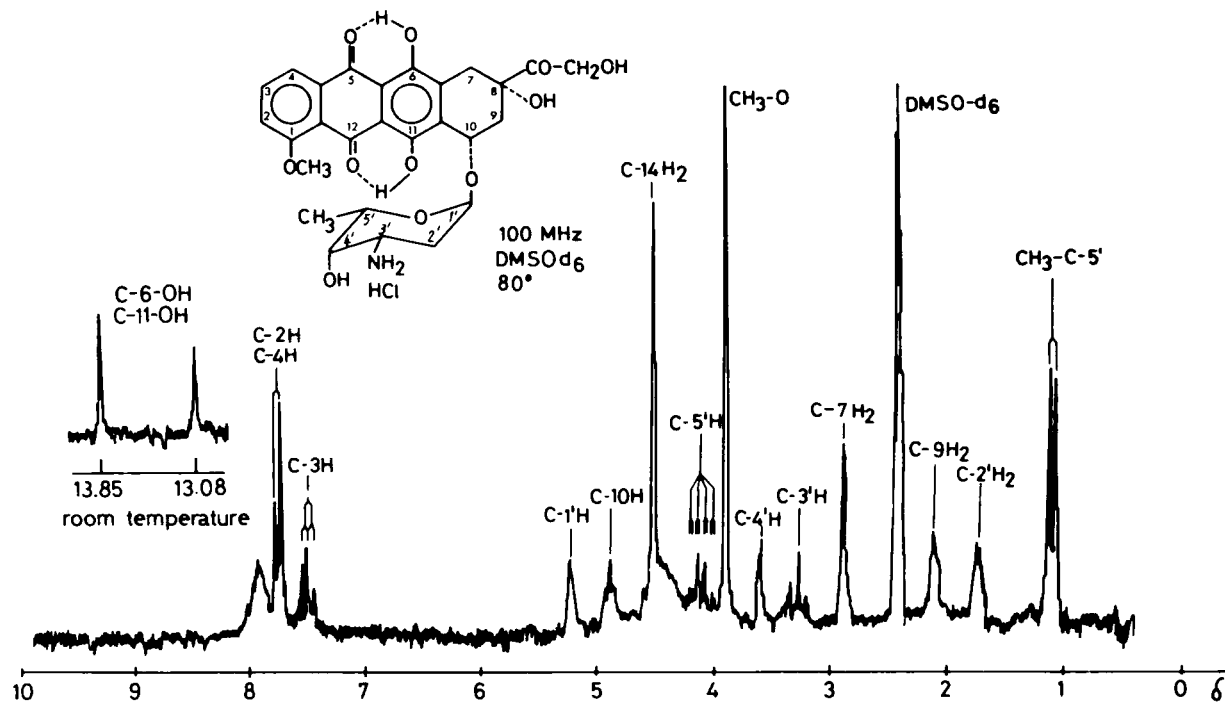


Figure 2. Proton NMR Spectrum of Doxorubicin



The  $^{13}\text{C}$  NMR spectra of doxorubicin hydrochloride, daunorubicin hydrochloride, the corresponding aglycones and of  $\alpha$ -methyl daunosaminide in  $\text{DMSO-d}_6$  solutions and the interpretation has been reported<sup>6</sup>. Figure 3 shows the FT  $^{13}\text{C}$  NMR proton noise-decoupled spectrum of doxorubicin hydrochloride in  $\text{D}_2\text{O}$  solution, recorded at 80 MHz on a Varian CFT-20 NMR spectrometer. Dioxane, which is not shown, was used as the internal standard. The interpretation of the spectrum is given in Table 3.

TABLE 3

$^{13}\text{C}$ -NMR data of doxorubicin hydrochloride in  $\text{D}_2\text{O}$  solution  
values (ppm) are referred to TMS.

Carbon	$\delta$	Carbon	$\delta$
1	161.0	4a	(134.5)
2	(119.2)	5a	111.0
3	137.3	6a	(134.0)
4	(120.2)	10a	(134.5)
5	(185.9)	11a	111.0
6	154.6	12a	(120.0)
7	32.8	$\text{CH}_3\text{O}$	57.2
8	76.6	1'	99.4
9	36.0	2'	28.5
10	69.0	3'	47.7
11	156.2	4'	(67.9)
12	(186.1)	5'	(67.0)
13	213.9	$\text{CH}_3\text{-5'}$	16.6
14	65.3		

Assignments with similar shift values  
given in parentheses may be interchanged.

### 2.3 Mass Spectra

The mass spectrum of doxorubicin hydrochloride itself cannot be obtained by electron-impact ionization, but this technique can be used to obtain the spectra of adriamycinone<sup>5</sup> and daunosamine<sup>7</sup>. A study of N-acetyl daunosamine derivatives has been published<sup>8</sup>. The mass spectra of some N-acylated daunorubicin derivatives have been published<sup>9</sup> and also the GC-MS of persilylated aglycone derivatives of doxorubicin and daunorubicin<sup>10</sup>.

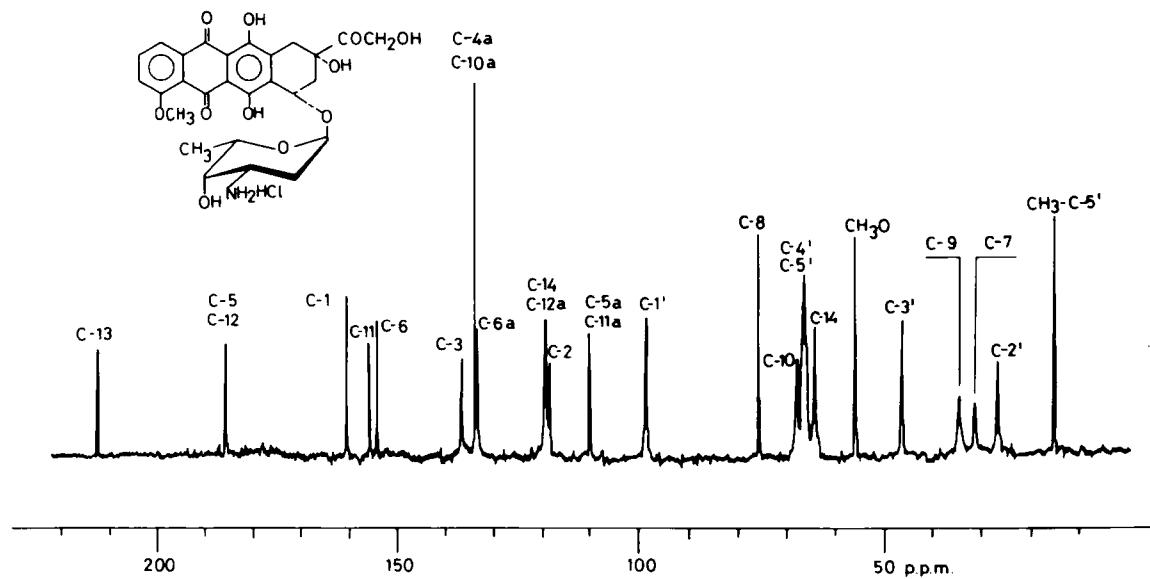


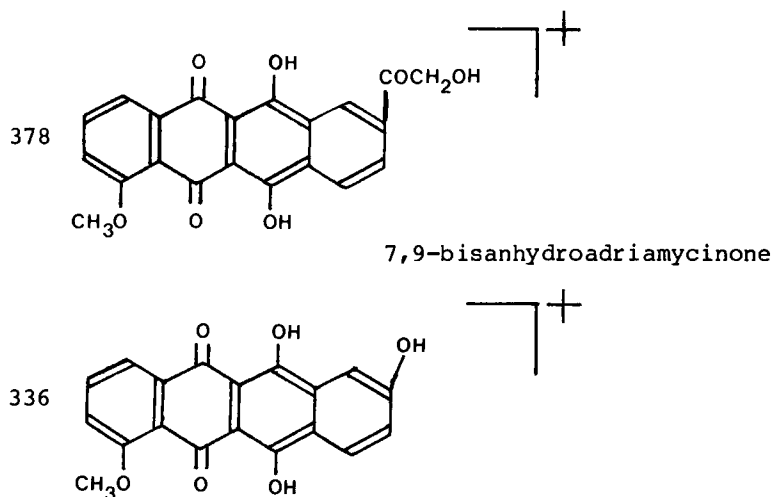
Figure 3.  $^{13}\text{C}$  NMR Spectrum of Doxorubicin

The intact molecule can be examined by field desorption ionization mass spectrometry<sup>11</sup>. Figure 4 shows the spectrum obtained on a Varian MAT311A spectrometer, equipped with a combined FD/FI/EI source (emitter heating current 20mA)<sup>12</sup>. Table 4 gives the assignments of the major fragmentation peaks.

TABLE 4

Field desorption mass spectrum of doxorubicin hydrochloride

<u>m/e</u>	<u>assignment</u>
544	M+1
543	Molecular ion
414	adriamycinone



#### 2.4 Ultraviolet and Visible Spectrum

The ultraviolet and visible spectrum of doxorubicin hydrochloride in methanol (c=1%) is shown in Figure 5<sup>13</sup>. The molecular absorptivities are given in Table 5.

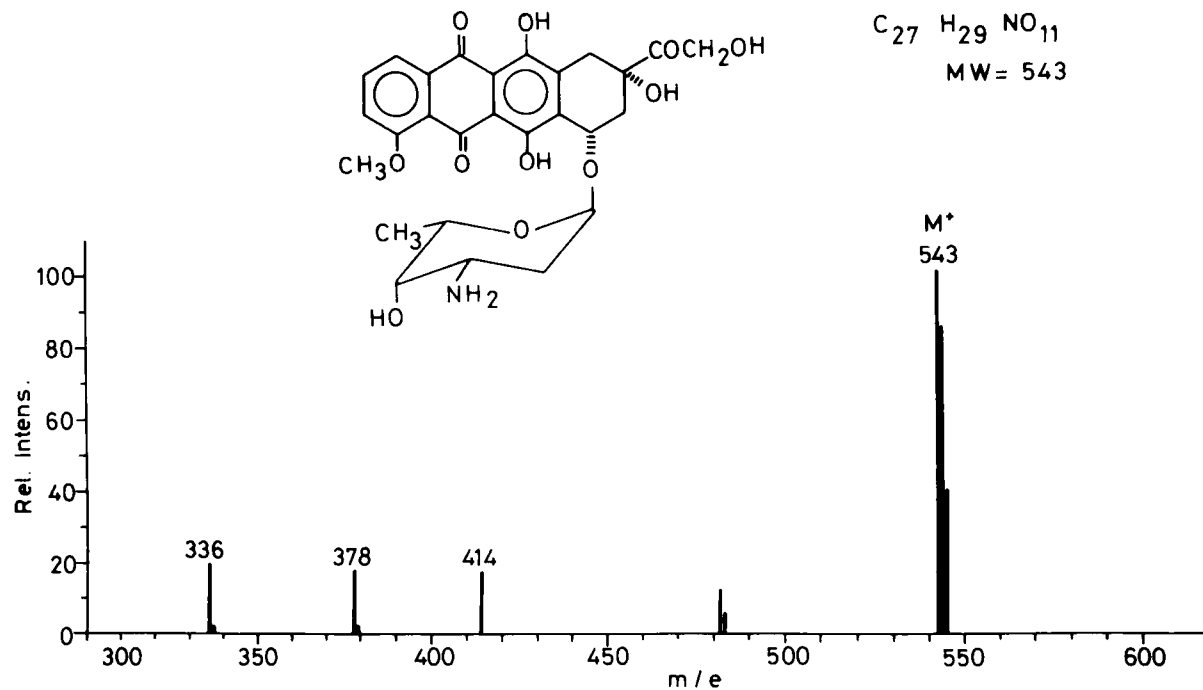


Figure 4. Field Desorption Mass Spectrum of Doxorubicin

TABLE 5Ultraviolet and Visible Molecular Absorptivities of  
Doxorubicin Hydrochloride in Methanol

<u>Wavelength</u>	<u>E</u>
233	38150
253	25500
290	8400
477	13050
495	13000
530	7200

2.5 Fluorescence Spectra

The fluorescence spectra of doxorubicin hydrochloride in water and ethanol at approximately 5 ppm, determined using a Perkin-Elmer MPF-2A spectrofluorimeter, are shown in Figure 6.<sup>13</sup>

2.6 Circular Dichroism

The chiral centers at C-8 and C-10 are responsible for the Cotton effects at 345 and 285 nm. The circular dichroism curves of methanol solutions of doxorubicin and daunorubicin hydrochlorides and of adriamycinone and daunomycinone in dioxane, determined using a Roussel-Jouan Dichrograph II, are shown in Figures 7 and 8. This technique has been used in the deduction of stereochemical relationships in the field of anthracyclines<sup>14</sup>.

2.7 Optical Rotation

The optical rotation  $[\alpha]_D^{25}$  of doxorubicin hydrochloride in methanol (0.1%) was determined at 589 nm using a Perkin-Elmer Model 241 MC polarimeter to be +255°.

2.8 Melting Point

Doxorubicin hydrochloride melts at 205°C with decomposition.

2.9 X-ray Diffraction

At this time no X-ray diffraction studies have been reported on doxorubicin hydrochloride or its derivatives.

Single crystal X-ray diffraction of N-bromoacetyl-daunorubicin solvate with acetone<sup>15</sup> confirmed the structure and absolute configuration of daunorubicin, which had previously been determined by chemical studies<sup>2,3,4</sup>. More recently these results were confirmed by an X-ray analysis of daunorubicin, as the hydrochloride

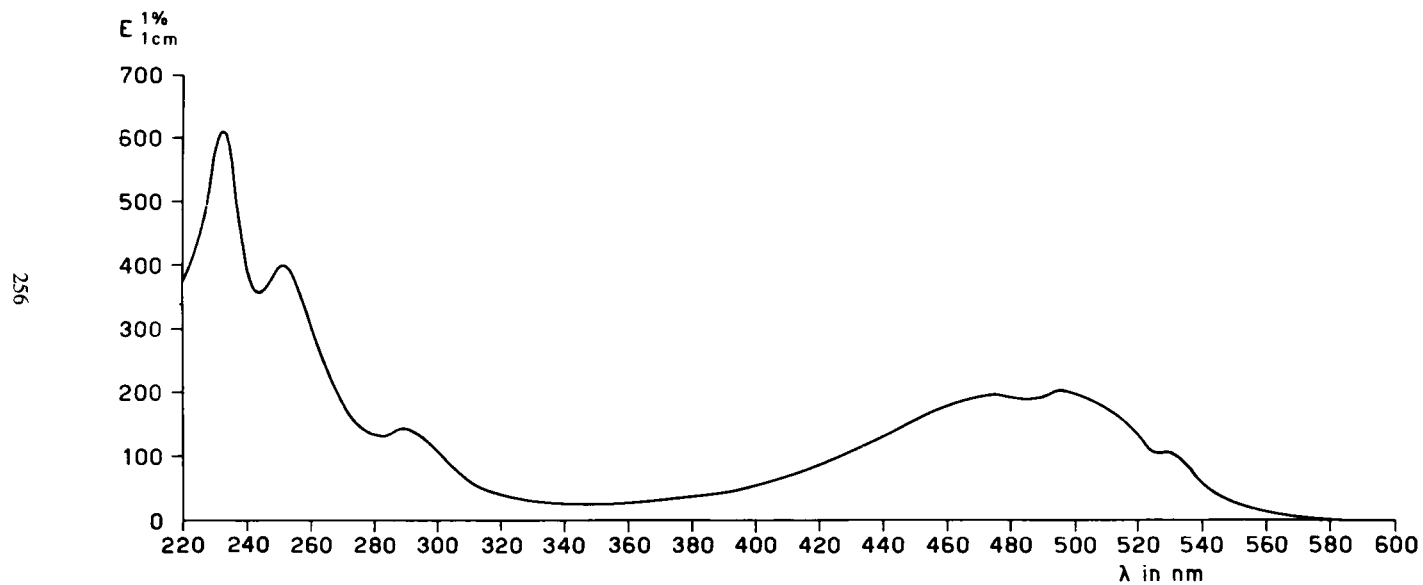


Figure 5. Ultraviolet and Visible Spectrum of Doxorubicin Hydrochloride in Methanol

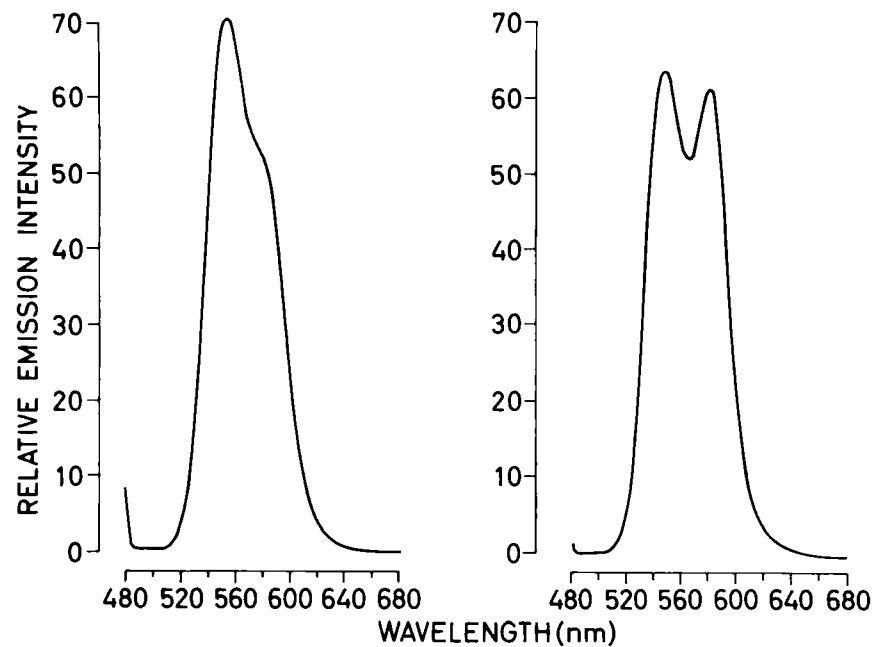


Figure 6. Fluorescence Spectra of Doxorubicin Hydrochloride in Water (left) and Ethanol (right). Concentrations approximately 5 mg/l.

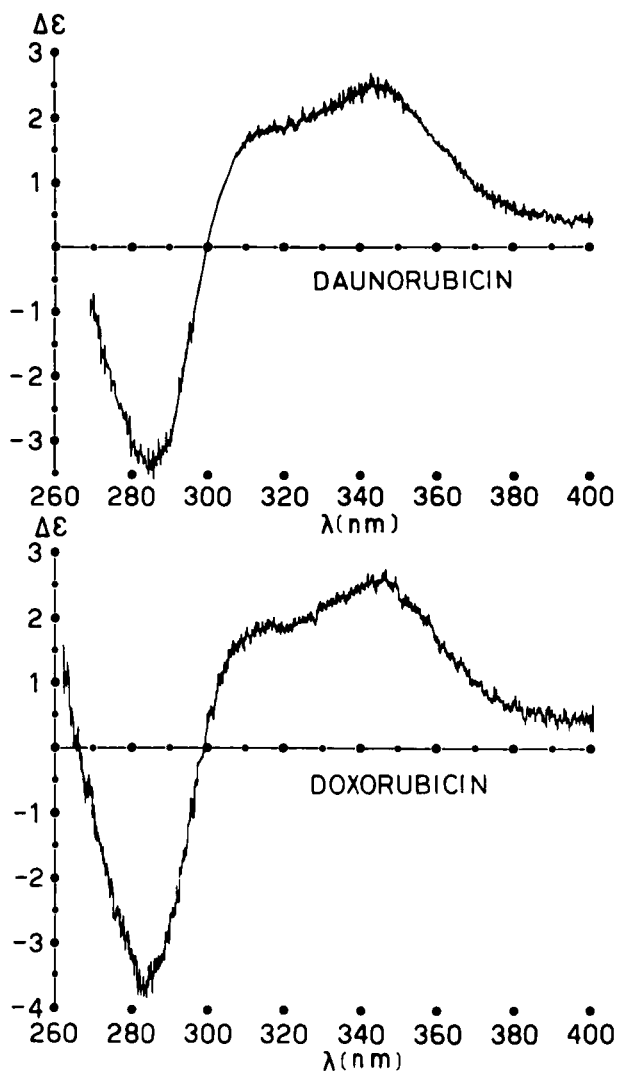


Figure 7. Circular Dichroism Curves of Doxorubicin and Daunorubicin Hydrochlorides in Methanol



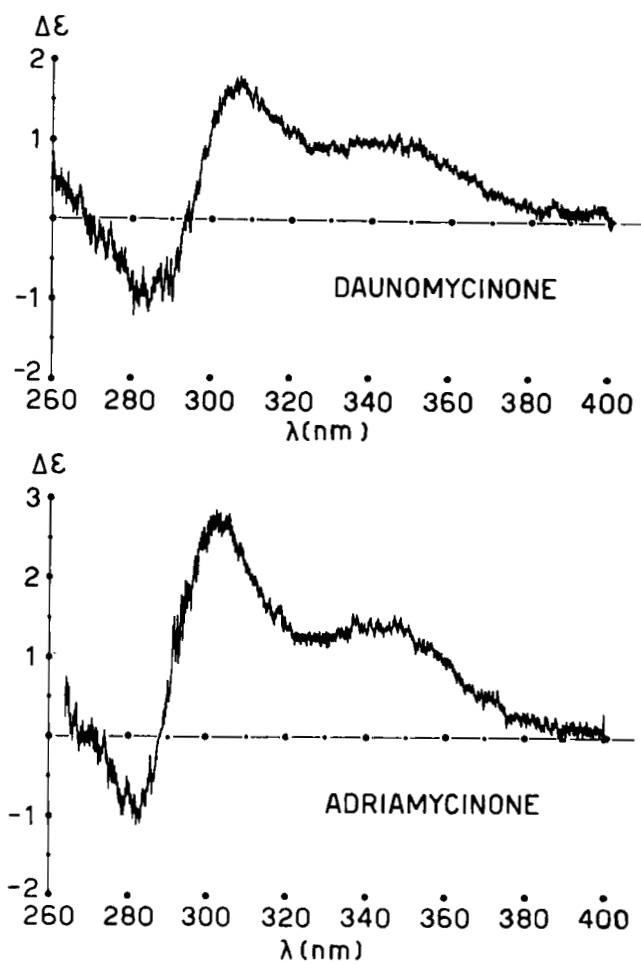


Figure 8. Circular Dichroism Curves of Adriamycinone and Daunomycinone in Dioxane Solution

monohydrate pyridine salt<sup>16</sup>. Some conformational differences were observed with respect to the N-bromoacetyl derivative.

### 2.10 Differential Scanning Calorimetry

The heating curve of doxorubicin hydrochloride obtained with a Perkin-Elmer Model DSC-1B scanning calorimeter at a temperature gradient of 8°C/min. is shown in Figure 9<sup>17</sup>. It shows an endotherm, corresponding to the solid-liquid transition at 202-205°C, partially superimposed by an endotherm due to decomposition which is at a maximum at 260°C and continues to higher temperatures.

### 2.11 Solubility

Doxorubicin hydrochloride is readily soluble in water, normal saline, methanol, acetonitrile and tetrahydrofuran, but only slightly soluble or insoluble in less polar organic solvents. The apparent partition coefficient ( $P_{app}$ ) between 1-octanol and Tris buffer at pH 7.0 with constant ionic strength ( $I = 0.1$ ) is 0.52 at room temperature (22-24°C) after shaking for 15 hours<sup>18</sup>.

### 2.12 Ionization Constant

A  $pK_a$  of 8.22 was determined for the hydrochloride with N/20 sodium hydroxide. Solutions of doxorubicin hydrochloride show indicator-like properties, turning from orange-red to blue-violet about  $pH = 9$ <sup>13</sup>. Values of -5.9, 8.2, 10.2, and 13.2 for  $pK_1$ ,  $pK_2$ ,  $pK_3$  and  $pK_4$ , determined by spectrophotometric methods, have been reported<sup>19</sup>.

### 2.13 Polarography

Due to its quinoidal system, doxorubicin gives characteristic polarograms at different pH values. These curves, determined using a Leeds-Northrup Electro-Chemograf type E polarograph, are shown in Figure 10<sup>13</sup>.

## 3. Synthesis

### 3.1 Microbiological

Doxorubicin can be obtained by aerobic fermentation of Streptomyces peucetius var. caesius followed by extraction with acidic acetone and purification by partition chromatography on a column of cellulose buffered at pH 5.4. The antibiotic is recovered from the eluates in 1-butanol saturated with pH 5.4 phosphate buffer by back extraction with dilute acid pH 3, followed by re-extraction into chloroform at pH 8.6. The chloroform solution is concentrated and doxorubicin crystallized as the hydrochloride on addition of an equivalent of methanolic

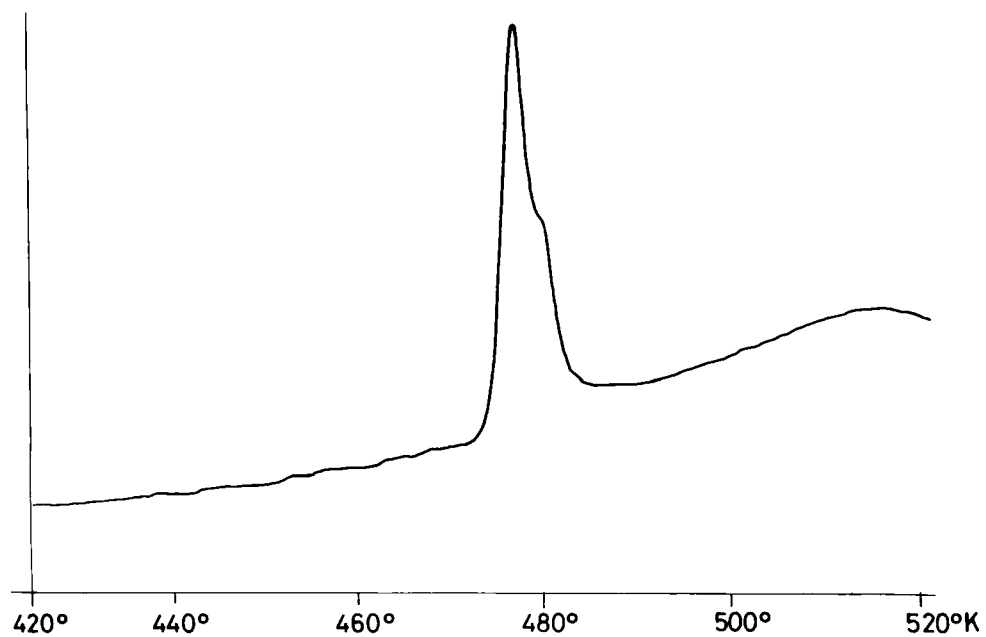


Figure 9. Differential Scanning Calorimetric Scan of Doxorubicin

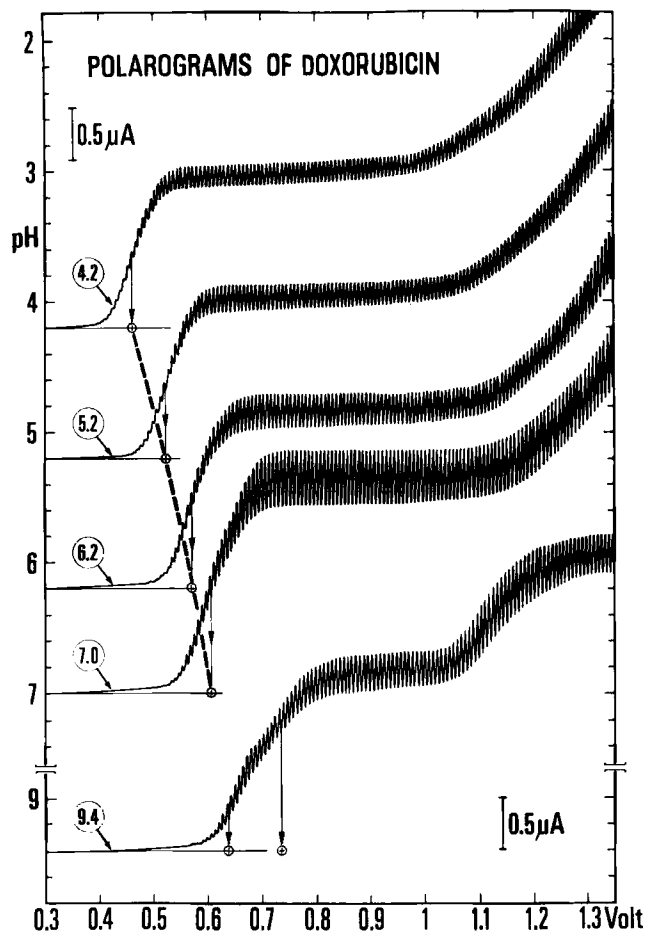


Figure 10. Polarograms of Doxorubicin in Solutions of Different pH Values

hydrogen chloride. Final purification is performed by crystallization from ethanol or from a methanol/1-propanol mixture<sup>20</sup>.

### 3.2 Chemical

Doxorubicin can be obtained<sup>21</sup> by reacting daunorubicin hydrochloride in a methanol/dioxane solvent mixture with a chloroform solution of bromine, forming 14-bromodaunorubicin. This is then hydrolyzed with an aqueous methanolic solution of sodium hydroxide under a nitrogen atmosphere. After dilution with water, the solution is extracted with chloroform and the organic extracts dried over anhydrous sodium sulfate, concentrated, treated with hydrogen chloride in anhydrous methanol, and then diluted with ethyl ether. The precipitate formed is doxorubicin hydrochloride, which is purified by crystallization from a mixture of methanol and 1-propanol. The above reaction pathway can be summarized as shown in Figure 11, in which the anthraquinone moiety is not shown.

### 4. Stability

Doxorubicin hydrochloride is very stable in the solid state. It has been stored for years at room temperature without any loss in potency or indications of degradation. The lyophilized powder of doxorubicin hydrochloride with lactose is also stable, if dry and stored in well closed containers at room temperature<sup>13</sup>. The active drug substance has also been found to be stable for three months at 60°C, and for three months in light of 500 ft. candles of illumination at room temperature. The lyophilized formulation is stable under similar lighting conditions, and at 60°C if the moisture content in the sealed vial is less than 1.0%.

The effect of pH values and buffer concentrations on the stability of aqueous solutions of doxorubicin hydrochloride has been determined by spectrophotometric and chromatographic methods. Doxorubicin is stable in acidic solutions in the pH range 3.0 to 6.5, but decomposes at increasing rates as the pH is increased from 6.5 to 12. Decomposition in aqueous solution gives complex mixtures of pigmented compounds with a wide range of chromatographic polarities. Apart from the isolation of adriamycinone from dilute acid solutions<sup>13</sup> the identification of the components of these mixtures has not been accomplished.

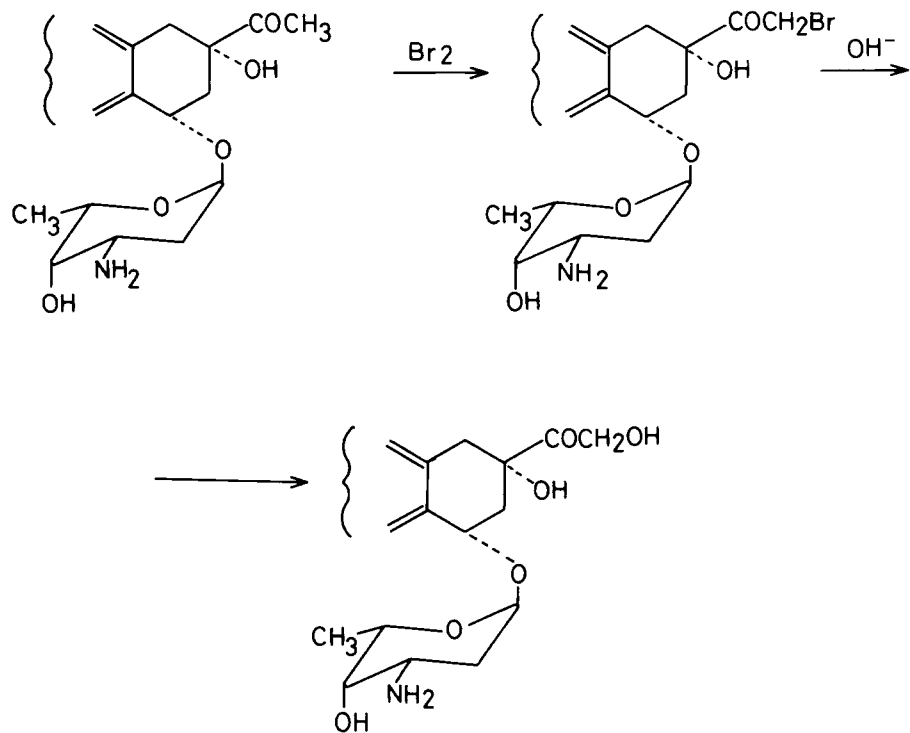


Figure 11. Synthetic Pathway for Doxorubicin

### 5. Metabolism

The two major metabolic transformations of doxorubicin in laboratory animals and in man are:

- a) The reduction of the side chain carbonyl group to a secondary alcohol, giving 13-dihydrodoxorubicin (adriamycinol).
- b) The reductive cleavage of the daunosamine moiety with the formation of 10-deoxyadriamycinone.

The first reaction is catalyzed by an enzyme named "daunorubicin reductase," an aldo-keto reductase of a very ubiquitous nature. The reductive splitting of the benzylic glycosidic bond is, on the contrary, rather unique as no other examples of enzyme catalysis of this otherwise chemically very facile reaction have been described<sup>1</sup>. The aglycone-like compounds thus formed are then further metabolized by other typical reactions such as O-methylation and conjugation<sup>22,23</sup>.

Doxorubicin and its metabolites extracted from the urine of patients treated with the drug were separated by chromatography on columns of silicic acid. The following compounds were isolated (in order of increasing polarity) (see Figure 12): 13-dihydroadriamycinone(3), 10-deoxy-13-dihydroadriamycinone(4), 1-demethyl-10-deoxy-13-dihydroadriamycinone(5), doxorubicin(1), 13-dihydrodoxorubicin(2), 1-demethyl-10-deoxy-13-dihydroadriamycinone-1-O-sulfate(6), 1-demethyl-10-deoxy-13-dihydroadriamycinone-1-O- $\beta$ -D-glucuronide(7). A total of 60% of the fluorescence in the urine was due to metabolites and the remainder was unchanged drug. As the recovery of doxorubicin fluorescence in bile and urine from a patient was about 60% of the administered dose, the authors pointed out the possibility of the presence of non-fluorescent metabolites<sup>24</sup>. The above mentioned metabolites were also detected in the plasma of patients under doxorubicin treatment<sup>25</sup>.

Protein binding studies using the ultracentrifugation method suggested that doxorubicin is bound to rabbit and human plasma proteins to an extent of 50%<sup>26</sup>, but a re-examination of the original Scatchard plot data changed this value to 90%<sup>27</sup>. Other studies using equilibrium dialysis have suggested complex binding relationships that need further investigation<sup>28</sup>.

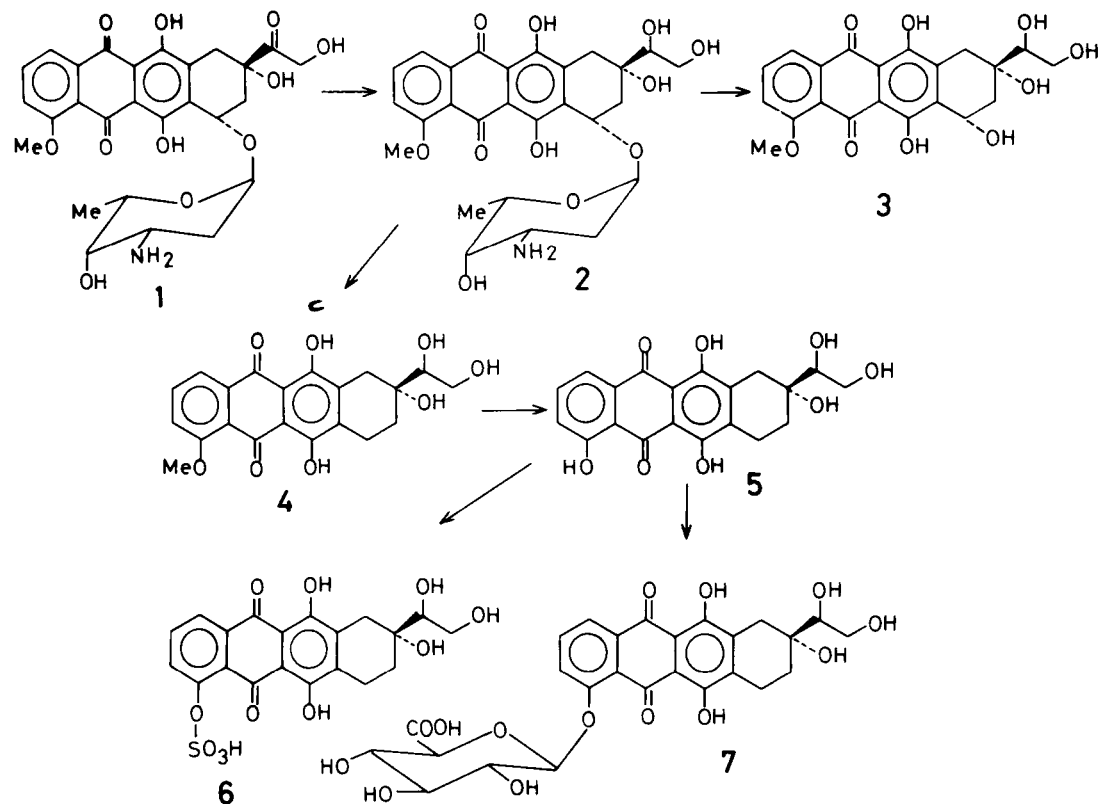


Figure 12. Urinary Metabolites of Doxorubicin



The main biochemical effects of doxorubicin are concerned with nucleic acid synthesis. The binding of this drug to DNA is considered responsible for the interference with template DNA function<sup>29</sup>. The DNA-doxorubicin binding constant has been determined to be approximately  $2 \times 10^6 \text{ M}^{-1}$  (30).

## 6. Methods of Analysis

### 6.1 Elemental Analysis

The elemental analysis of doxorubicin hydrochloride (Farmitalia reference standard batch GDA 1) is as follows:

	<u>% Theory</u>	<u>% Found</u>
C	55.91	56.08
H	5.22	5.33
N	2.41	2.16
Cl	6.11	5.85

### 6.2 Spectrophotometric Analysis

The visible absorption maximum at 495 nm ( $E_{1\text{cm}}^{1\%} = 223$ ) can be used for the quantitation of doxorubicin in dosage forms.

The fluorescence properties of doxorubicin can be used for the determination of total anthracycline at low concentrations<sup>31</sup>.

### 6.3 Electrochemical Analysis

Chronopotentiometric<sup>31</sup>, cyclic voltammetric<sup>32</sup> and polarographic<sup>32,33</sup> assays of doxorubicin hydrochloride have been reported. These techniques determine the total anthracycline content.

### 6.4 Paper Chromatography

Doxorubicin can be separated from the aglycone, adriamycinone, by paper chromatography using either of the following two systems<sup>13</sup>.

- A) 1-butanol saturated with pH 5.4 M/15 phosphate buffer.
- B) 1-propanol/ethyl acetate/water, 7/1/2 by volume.

The  $R_f$  values for doxorubicin and adriamycinone are 0.1 and 0.3, and 0.25 and 0.65 for systems A and B respectively.

### 6.5 Thin Layer Chromatography

Thin layer chromatographic systems for doxorubicin are given in Table 6.

**TABLE 6**

Thin layer chromatographic systems for doxorubicin.

<u>Adsorbent</u>	<u>Solvent System</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
Silica gel	Methylene chloride/ methanol/water (100/20/2)	0.17	13
Silica gel	1-butanol/acetic acid/water (4/1/5)	0.33	13
Silica gel	Chloroform/95% ethanol/ trifluoroacetic acid (75/20/5)	0.23	34
Silica gel	Chloroform/methanol/acetic acid (93/5/2, plate dried, then 76/20/4)	0.2	35
Silica gel sprayed with phosphate buffer (pH = 7.0)	Chloroform/methanol/water (140/60/10)	0.3	36
Polyamide/ cellulose	1-Butanol/2-propanol/isopropyl ether/acetic acid/water (35/6/6/9/44)	0.3	37

### 6.6 Liquid Chromatography

Liquid chromatographic systems for doxorubicin hydrochloride are given in Table 7.

TABLE 7Liquid chromatographic systems for doxorubicin

<u>Stationary Phase</u>	<u>Mobile Phase</u>	<u>Approximate Doxorubicin Capacity Factor (k')</u>	<u>Ref.</u>
Silica (5 micron)	2-propanol/isopropyl ether/0.125 M acetate buffer pH 4.5 (65/30/5)	10	38
Silica (5 micron)	2-propanol/0.5 sodium acetate buffer pH 4.5 (96.2/3.8)	4	39
Silica (5 micron)	Methylene chloride/methanol/25% ammonia/water (90/9/0.1/0.8)	4	40
Cyanopropylsilica (10 micron)	Chloroform/methanol/acetic acid/water (79.8/14.1/4.7/14)	3	41
Cyanopropylsilica (10 micron)	Chloroform/methanol/water (96/5/1)	-	42
Corasil-phenyl (37-75 micron)	Linear gradient, 16% acetonitrile/in water to 20% acetonitrile/80% pH 4 formate buffer	10	43
Corasil-phenyl (37-50 micron)	Linear gradient 0 to 40% acetonitrile in pH 4.0 ammonium formate buffer	10	44
Octadecyl-silica (10 micron)	Methanol/aqueous solution of PIC B-7 (heptane-sulfonic acid) (50/50)	14	45
Octadecyl-silica (10 micron)	Acetonitrile/aqueous phosphoric acid pH 2, (31/69)	3	46
Octadecyl-silica (10 micron)	Methanol/water/acetic acid (66/33.2/0.8)	1.4	47
Octyl-silica (10 micron)	Acetonitrile/10 <sup>-2</sup> M. aq. phosphoric acid (40/60)	2	48

### 7. Determination of Doxorubicin in Biological Fluids

Total anthracycline compounds in biological fluids can be determined by fluorimetric methods<sup>31,49</sup>. Radioimmunoassay procedures have also been reported<sup>50</sup>. The emphasis is now on the separate determination of metabolites and intact drug in biological fluids. One such method coupled liquid chromatography followed by RIA<sup>43</sup> but it was rather time-consuming. TLC followed by fluorescence scanning has been reported<sup>35</sup> and used for disposition predictions<sup>27</sup>.

The most recently published methods have used reversed-phase liquid chromatography<sup>51,52</sup> or normal phase liquid chromatography<sup>51,53</sup> with fluorescence detection. These methods have been applied to tissue distribution studies<sup>51</sup>. Similar methods<sup>54</sup> used for daunorubicin and metabolites should also be applicable.

### 8. Analysis of Pharmaceutical Formulations

The identification and/or determination of doxorubicin hydrochloride in Adriamycin involves the use of visible spectrophotometry, thin layer chromatography followed by spectrophotometry or microbiological agar diffusion<sup>55</sup>. However, the recently published liquid chromatographic procedure<sup>46</sup> is replacing the above physical methods.

### 9. Miscellaneous

Pharmaceutical preparations of doxorubicin hydrochloride, trade-marked Adriamycin, have been patented<sup>20</sup>.

### 10. Acknowledgments

Acknowledgment is made to Drs. F. Arcamone and S. Penco of Farmitalia-Carlo Erba SpA. and Drs. G. Davis, W. Hausmann and J. Short of Adria Inc., for their useful advise during the preparation of the manuscript.

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# FLUPHENAZINE DECANOATE.

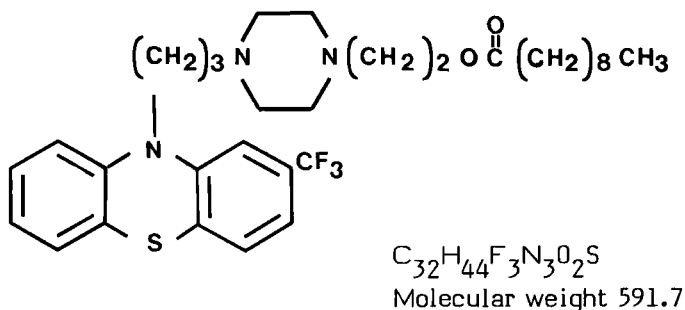
*Geoffrey Clarke*

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## 1. Description

### 1.1. Name, Formula, Molecular Weight.

Fluphenazine decanoate is 4-[3-[2-(trifluoromethyl)phenothiazin-10-yl]propyl]-1 piperazine ethanol decanoate ester; Prolixin decanoate; SQ 10,733



### 1.2. Appearance, Odor, Color.

Fluphenazine decanoate is a pale yellow to yellow orange viscous liquid with a characteristic odor. At room temperature the liquid will slowly crystallise.

## 2. Physical properties

### 2.1 Infrared spectrum

The infrared spectrum of fluphenazine decanoate (lot 117102, purity 99%) in the liquid phase (as a thin film) is given in figure 1. The following assignments have been made for the most characteristic bands (37).

Frequency( $\text{cm}^{-1}$ )	Assignment
2940	Aromatic C-H stretching vibrations
1740	Ester carbonyl stretching vibrations
1605 1575	aromatic ring skeletal vibrations
930,870 820,750	C-H out of plane bending vibrations

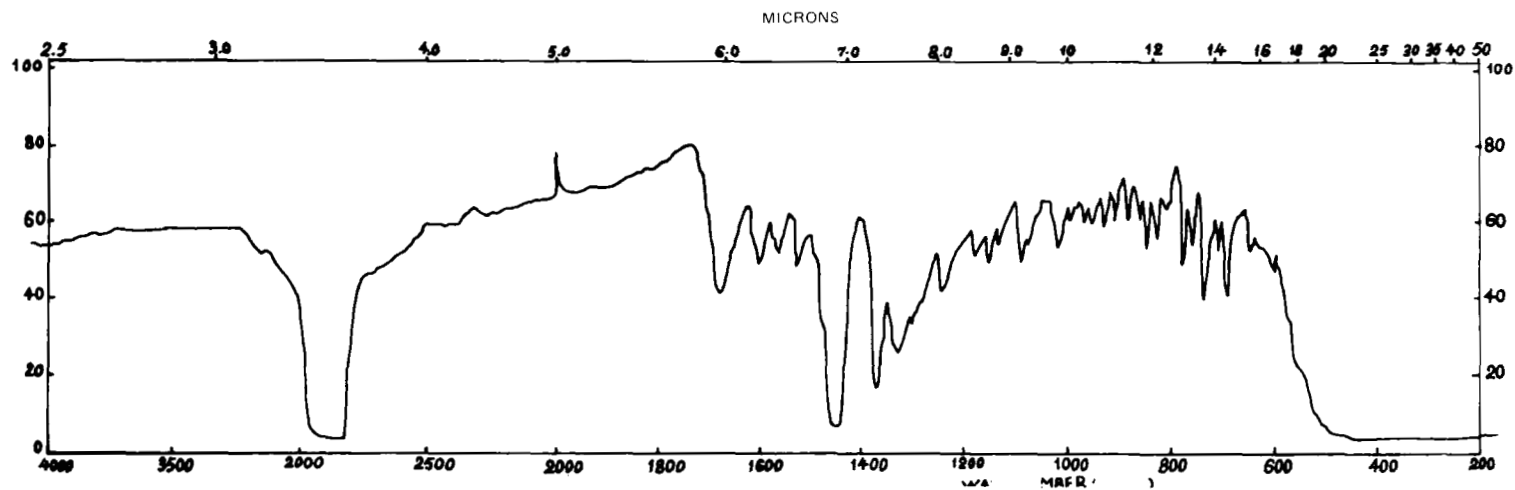


Figure 1. Infrared spectrum of Fluphenazine decanoate as a thin film.  
Instrument: Unicam SP 1000.

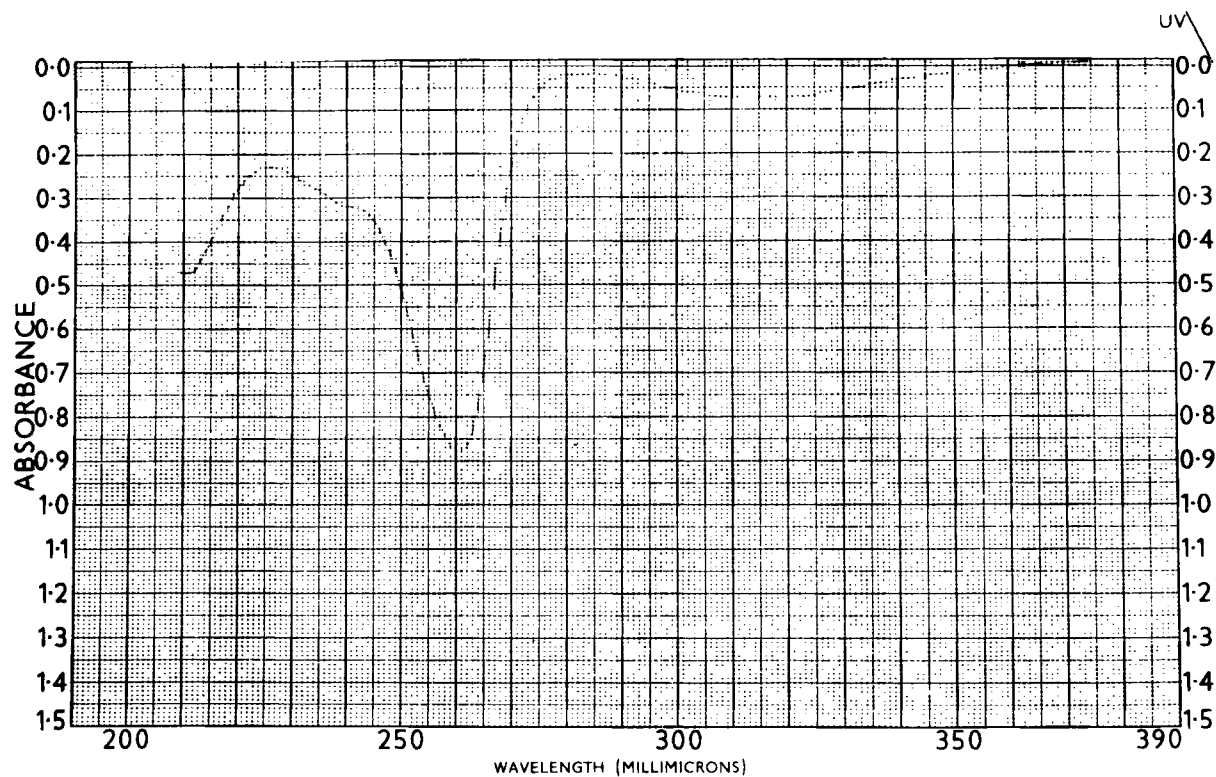


Figure 2. Ultraviolet spectrum of Fluphenazine decanoate in methanol (15ug ml<sup>-1</sup>). Instrument: Perkin Elmer 137.

The four bands between  $750$  and  $930\text{cm}^{-1}$  are reported to be characteristic of 2, 10 disubstituted phenothiazines and the two bands at  $1605$  and  $1575\text{cm}^{-1}$  characteristic for phenothiazines in general<sup>(37)</sup>.

## 2.2. Ultraviolet spectrum

The ultraviolet spectrum of fluphenazine decanoate (lot 117102, purity 99%) in methanol is given in figure 2. Similar spectra are obtained in ethanol and chloroform although the shoulder at  $240\text{nm}$  is obscured in chloroform due to absorption of the solvent. The spectrum given in figure 2 is characteristic of a phenothiazine and the location of the most intense peak at  $261\text{nm}$  is consistent with a halogen substituent in the 2-position<sup>(37)</sup>.

	$E_{1\text{cm}}^{1\%}$		
	$240\text{nm}$	$261\text{nm}$	$315\text{nm}$
Chloroform	-	551	74.3
Methanol	220	562	65.9
Ethanol(95%)	221	586	68.4

## 2.3 Fluorescence spectrum

Fluphenazine decanoate does not exhibit any significantly measurable fluorescence in ethanolic solution. Fluorescence measurement can be made however after prior oxidation to the sulfoxide. (See section 6.5).

## 2.4 Nuclear magnetic resonance spectrum.

The  $100\text{ MHz}$  spectrum of fluphenazine decanoate in  $\text{DMSO-}d_6$  (internal reference TMS) is given in figure 3.

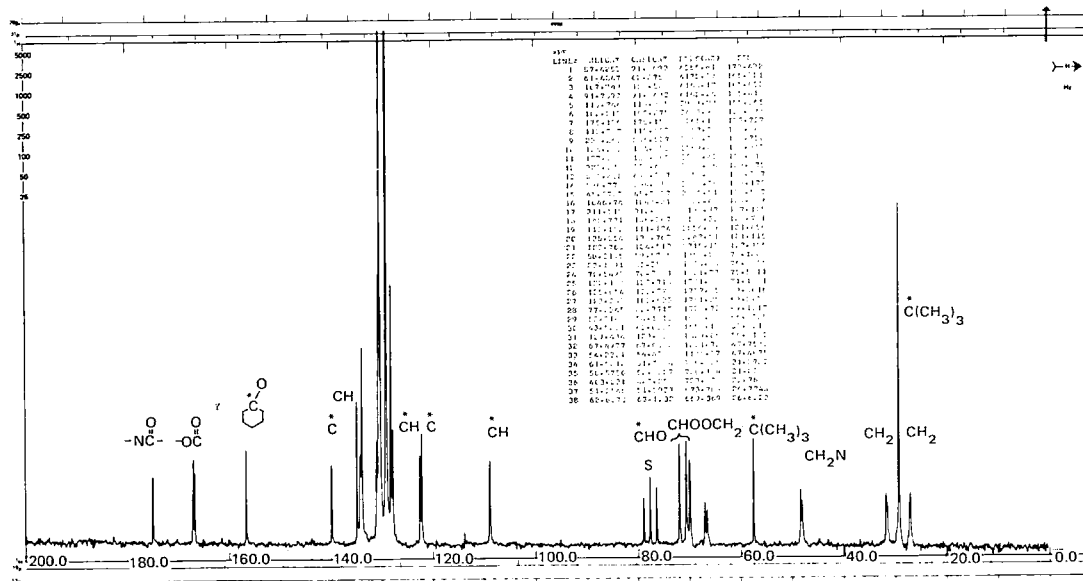


Figure 3. Nuclear magnetic resonance spectrum of Fluphenazine decanoate in DMSO-d6. Instrument: Thompson Packard.



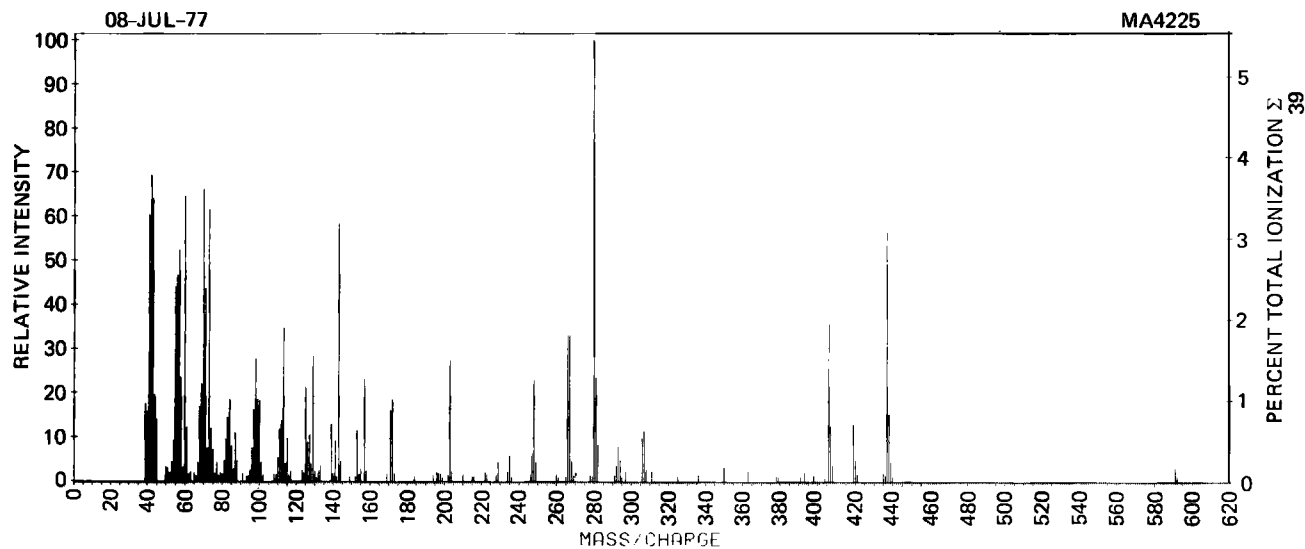


Figure 4. Low resolution Mass Spectrum of Fluphenazine decanoate. Instrument: MS-9.



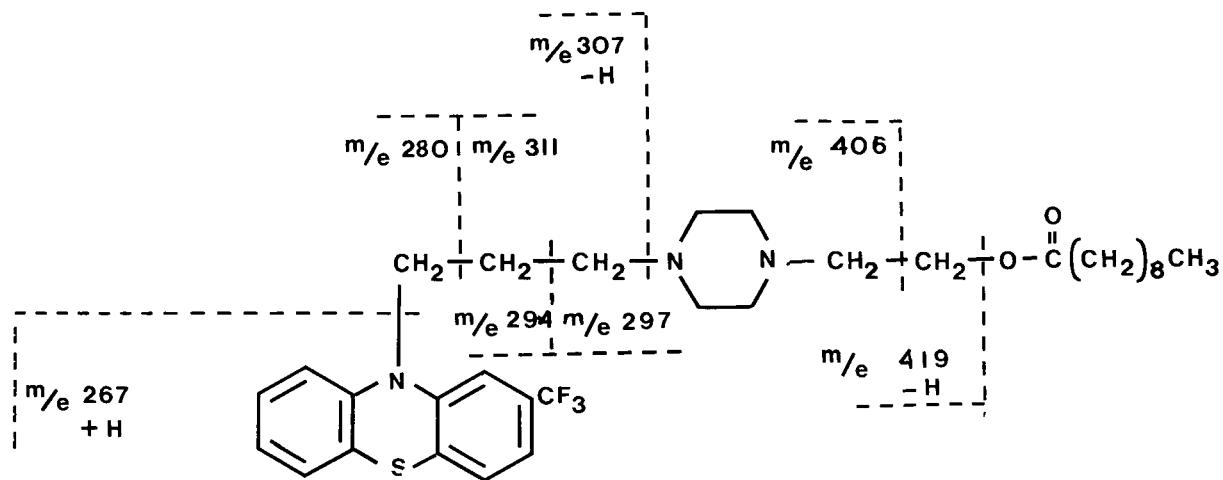


Figure 5. Fragmentation of Fluphenazine decanoate.

## 2.9 pKa

The pKa and pKa<sub>2</sub> values for fluphenazine decanoate have not been reported. However they would be expected to be very similar to those reported for fluphenazine enanthate of about 3.4 and 8.0.(see analytical profiles of drug substances, volume 2).

## 2.10 Differential thermal analysis

Between 15°C and 200°C only the endotherm due to melting at 30°C is observed for the crystalline material. The dihydrochloride salt gives an endotherm due to melting at 180°C.<sup>(3)</sup>

## 3. Synthesis

Fluphenazine decanoate(I) can be prepared from fluphenazine(II) by refluxing a chloroform solution of (II) with decanoyl chloride. (See Figure 6). The fluphenazine decanoate is extracted as the hydrochloride salt and recrystallised from a mixture of anhydrous acetone and ether. After reconversion to the base with aqueous sodium carbonate the fluphenazine decanoate is extracted into ether, dried and concentrated by evaporation<sup>(4,10)</sup>.

## 4. Stability

Fluphenazine decanoate(I) will hydrolyse in alkaline medium to fluphenazine(II)<sup>(5)</sup>. In the presence of peroxides, oxidation of the piperazine nitrogen to an N-oxide(III) occurs probably by a free radical mechanism.<sup>(5,6,7)</sup> Fluphenazine decanoate will undergo photolysis to form a sulphoxide(IV)<sup>(5)</sup>. (See Figure 6).

## 5. Drug metabolism

Studies with <sup>14</sup>C labelled fluphenazine decanoate in the dog have been reported. The fluphenazine decanoate is hydrolysed to fluphenazine by plasma esterases and excreted in the urine. The metabolism of fluphenazine decanoate, in the dog, is therefore similar to that of fluphenazine dihydrochloride and fluphenazine enanthate<sup>(8)</sup>. (See analytical profiles of drug substances, volume 2). Traces of residual fluphenazine decanoate and/or its metabolites were found in the lung, liver, kidney, skin and heart of the dog but none in the brain<sup>(8)</sup>.

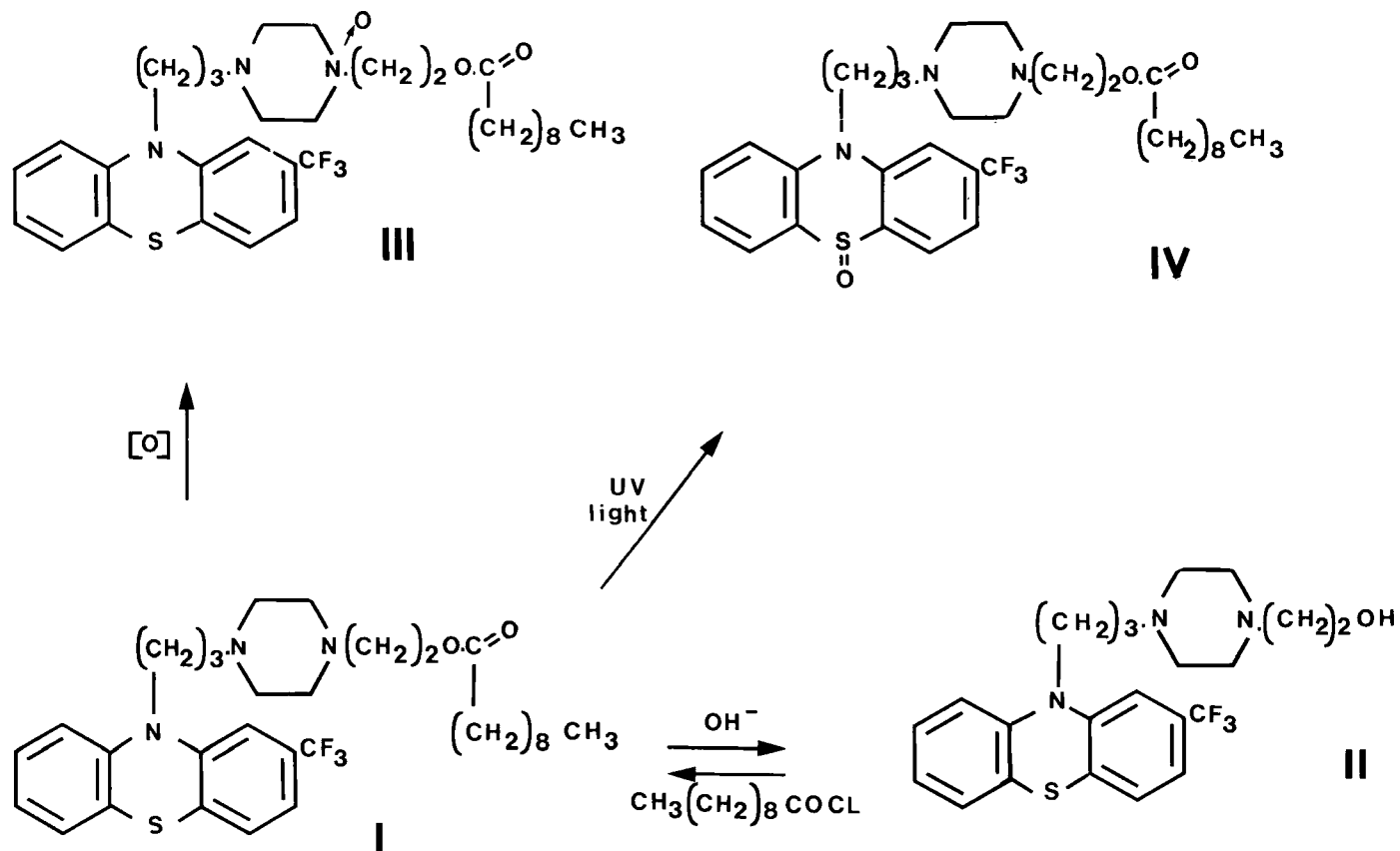


Figure 6. Chemistry of Fluphenazine decanoate.

## 6. Methods of analysis

### 6.1 Elemental analysis

The following analysis has been made<sup>(10)</sup>

	Calculated	Found
C	64.94	65.19
H	7.42	7.68
N	7.09	7.29

### 6.2 Non-aqueous titration

Fluphenazine decanoate can be titrated with perchloric acid in glacial acetic acid using crystal violet as the indicator. The neutralisation equivalent is 295.75. End point detection either visually with malachite green or potentiometrically has also been reported<sup>(9)</sup>.

### 6.3 Spectrophotometric analysis

The UV absorbance at 261nm of fluphenazine decanoate in methanol can be used as a quantitative assay. UV spectrophotometry however will only differentiate between fluphenazine decanoate and its sulfoxide, therefore a chromatographic separation of fluphenazine decanoate from other related substances usually precedes UV measurement(See section 6.6).

### 6.4 Colorimetric analysis

Fluphenazine decanoate can be extracted as an ion-pair with bromothymol blue into toluene from a pH 2.55 glycine/HCl buffer. The absorbance of the solution is measured at 400nm.<sup>(1)</sup>

A solution of fluphenazine decanoate in ethyl acetate when shaken with a pH2 buffered solution of palladium chloride will form a complex. This complex is soluble in the ethyl acetate phase and can be measured at 440nm.<sup>(12,13)</sup> Palladium complexes of any other esters of fluphenazine present as trace impurities, would also be formed in the ethyl acetate phase. However, the major hydrolysis product fluphenazine forms an aqueous soluble complex and would remain in the buffer phase. Therefore, spectrophotometric measurement of the aqueous buffer phase could be used as an assay for fluphenazine in fluphenazine decanoate<sup>(12)</sup>.

### 6.5 Fluorimetric analysis

A spectrofluorimetric procedure has been reported in which a solution of fluphenazine decanoate in methanol/sulphuric acid (80:20), after oxidation with ceric ions to the sulphoxide, fluoresces at 400nm when activated at 343nm.<sup>(14)</sup>

### 6.6 Chromatographic analysis

#### 6.6.1 Column chromatography

Fluphenazine decanoate and related substances can be adsorbed onto a column of silica gel from a chloroform solution. The fluphenazine decanoate can be selectively eluted from the column with a solvent mixture of cyclohexane/methanol/methylacetate (67.2:35.6:97.2). After removing the solvent by evaporation the fluphenazine decanoate can be quantified by dissolving in methanol and measuring the UV absorbance at 261nm<sup>(15)</sup>. The major degradation products, fluphenazine and fluphenazine decanoate N-oxide are not eluted from the silica gel column.

#### 6.6.2 Paper chromatography

The following systems have been reported, although no  $R_f$  values are quoted. Benzene/acetic/water(2:2:1) descending on Whatman No.1 paper and sodium formate(1 molar) ascending on Whatman 3MM paper for the separation of fluphenazine decanoate, fluphenazine and fluphenazine sulphoxide. Methanol/water(85:15) descending on Whatman No.1 paper impregnated with castor oil(2% in ether) for the separation of fluphenazine decanoate, fluphenazine, fluphenazine octanoate and fluphenazine dodecanoate<sup>(16)</sup>. Location is by UV light and quantitation by elution with 95% ethanol and measuring the absorbance at 261nm.

#### 6.6.3 Thin layer chromatography

A summary of the solvent systems and separations reported is given in table 1. The adsorbent used in all systems is silica gel G with a fluorescent indicator. Location of separated compounds is made by fluorescence quenching of UV light(366 or 254nm) or colorimetrically by spraying with 50% sulphuric acid to produce red zones. The solvent systems referred to in table 1 are as follows

Solvent System	Fluphenazine	Fluphenazine sulphoxide	R <sub>f</sub> values		
			Fluphenazine decanoate N-oxides	Fluphenazine decanoate	Fluphenazine decanoate sulphoxide
I	0.60	0.25	0.00, 0.16	0.80	0.73
II	0.73	-	0.00, 0.20* 0.08, 0.25	0.84	0.80
III	0.10	0.0	0.0	0.50	0.48
IV	0.10	0.0	0.0	0.80	0.75
V	0.80	-	-	0.90	-

\*All 4 zones have not been positively identified as N-oxides.

Table 1. Thin layer chromatography.

- I Cyclohexane/acetone/ammonia(30:80:5)<sup>(17)</sup>
- II Chloroform(saturated with ammonia)/methanol(80:2)<sup>(18)</sup>
- III Cyclohexane/acetone/ammonia(36:60:0.6)<sup>(17)</sup>
- IV Methanol/ethyl acetate/cyclohexane/chloroform<sup>(19)</sup>  
(9:25:17:38)
- V Chloroform/methanol/ammonia(9:10:0.5)<sup>(20)</sup>

Solvent system (III) has been used as the basis for a quantitative assay, the separated zones being eluted with methanol and the UV absorbance measured at 261nm<sup>(21)</sup>

#### 6.6.4 Gas liquid chromatography

Fluphenazine decanoate has been separated from fluphenazine by chromatographing the silylated mixture on a 5' x  $\frac{1}{8}$ " column of 3% JXR\* on Gas Chrom Q at 280°C. The carrier gas was nitrogen and detection was by flame ionisation. Perphenazine was used as an internal standard and the following retention times were report.<sup>(22)</sup>

Fluphenazine	4 minutes
Perphenazine	7 minutes
Fluphenazine decanoate	24 minutes

The silylation procedure was necessary in the above method to satisfactorily chromatograph the fluphenazine. However, fluphenazine esters do not require silylating and two other procedures have been reported for the separation of fluphenazine decanoate from other fluphenazine esters. With the exception of the temperature and the absence of silylation the conditions were as above. The following separations were reported.

	305°C <sup>(23)</sup>	330°C <sup>(24)</sup>
Fluphenazine octanoate	5.2 minutes	
Fluphenazine decanoate	7.4 minutes	1 minute
Fluphenazine dodecanoate	11.2 minutes	
Fluphenazine stearate/ oleate/linoleate		3.4 minutes

\*JXR-Applied Science Laboratories Inc.State College P.A.U.S.A.

### 6.6.5 High performance liquid chromatography

A reversed phase HPLC system for the separation of fluphenazine from fluphenazine decanoate and other fluphenazine esters has been reported.<sup>(25)</sup> The effects of the pH of the mobile phase and the chain length of the stationary phase were studied and the following separation reported.

Column	:	Partisil-TMS*(trimethylsilane) 200 x 4.6mm ID
Mobile phase	:	Methanol/acetonitrile/ 1% ammonium carbonate (1:1:0.3)
Flow rate	:	2ml min <sup>-1</sup>
Detection	:	UV at 260nm
Retention times	:	Fluphenazine 2.6 minutes
		Fluphenazine decanoate 3.3 minutes
		Fluphenazine myristate 4.2 minutes
		Fluphenazine palmitate 4.8 minutes
		Fluphenazine stearate 5.8 minutes

Slight variation in the ammonium carbonate concentration had little effect over the range 0.1-1%. However, changes in the ratio of total organic phase to aqueous phase has a marked effect on retention times<sup>(25)</sup>. Hence the following separation has been reported<sup>(26)</sup>.

Column	:	Partisil-TMS* 250 x 4.6mm ID.
Mobile phase	:	Methanol/acetonitrile/ 0.45% ammonium carbonate (1:1:1)
Flow rate	:	2ml min <sup>-1</sup>
Detection	:	UV at 260nm.
Retention times	:	Fluphenazine 4.5minutes
		Fluphenazine decanoate a) 9 minutes
		N-oxides b) 11 minutes
		Fluphenazine decanoate 14.5 minutes



Using a similar mobile phase to the above a separation of the octanoate and dodecanoate impurities in fluphenazine decanoate has been reported<sup>(27)</sup>.

Column	:	Bondapak C18** 300 x 3.9mm ID
Mobile phase	:	Methanol/acetonitrile/ 0.4% ammonium carbonate (1:1:0.5).
Flow rate	:	2ml min <sup>-1</sup>
Detection	:	254nm
Retention times	:	Fluphenazine 2 minutes Fluphenazine octanoate 3 minutes Fluphenazine decanoate 4.5 minutes Fluphenazine dodecanoate 6.5 minutes

\*Partisil, Reeve Angel, Clifton NJ. US.A.

\*\*Bondapak, Waters Associates.

## 7. Body fluid and tissue analysis

The metabolism of fluphenazine decanoate is mainly that of its hydrolysis product fluphenazine. Whilst traces of the unhydrolysed fluphenazine decanoate have been detected by <sup>14</sup>C tracing,<sup>(28)</sup> most of the reported work has been directed towards the detection and estimation of fluphenazine and its metabolites. In man, blood plasma levels of fluphenazine have been determined by <sup>14</sup>C tracing<sup>(28)(34)</sup>, after initial extraction of the alkaline plasma with n-heptane. Further partitioning<sup>(34)</sup> separated various metabolites in the urine and faeces<sup>(34)</sup>. Urine and plasma extracts have also been analysed by GLC<sup>(34)</sup> using an alkali-bead nitrogen sensitive detector and a column of 3% OV-17\* on Chromosorb W at 215°C. A similar procedure using a flame ionisation detector has also been reported<sup>(30)</sup>. Another GLC procedure has been reported<sup>(29)</sup> for determining fluphenazine and its metabolites in urine. The urine was extracted by adsorption onto an Amberlite XAD-2 column, washing with pH8.5 ammonium chloride buffer and elution with methanol. The extracted metabolites were chromatographed as their trimethyl silyl derivatives on 2% SE-30 on Gas Chrom Q at 225°C. Detection was by flame ionisation.

A fluorimetric procedure for blood plasma has been reported in which the plasma is extracted with heptane/isoamyl alcohol(98.3:1.7) after alkaline hydrolysis at 100°C<sup>(31)</sup>. The metabolites are back extracted into 0.1M phosphate buffer and oxidised with hydrogen peroxide. Fluorescence measurement was at 405nm, exciting at 350nm.

An HPLC procedure has been reported for hexane extracted serum using a glassy carbon electrode as an electrochemical detector<sup>(35)</sup>. The mobile phase was methanol/0.05M phosphate buffer, pH 6.9(53:47) and the separation was achieved on a Lichrosorb S1(60)column(Merck, Darmstadt, GFR), treated with dichlorodimethylsilane.

A TLC separation of metabolites in animal tissues has been reported<sup>(36)</sup>. The tissues were extracted with dichloromethane and chromatographed on silica gel in chloroform/isopropyl alcohol(10:1) and isopropyl alcohol/chloroform/ammonia/water (32:16:2:1).

\*OV-17, Applied Science Laboratories Inc.,  
State College PA U.S.A.

### Acknowledgement

The author wishes to thank Mrs. M. Watson for her invaluable secretarial help.

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# GENTAMICIN SULFATE

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## Gentamicin Sulfate

### 1. Description

#### 1.1 Drug Properties

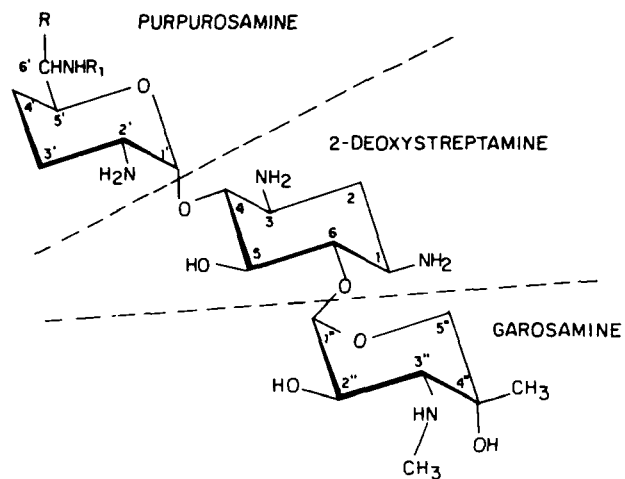
Gentamicin is an important member of the aminoglycoside class of antibiotic substances that was first isolated in 1963 by Weinstein *et al.*<sup>1</sup> from two previously undescribed species of *Micromonospora*. Isolation and preliminary chemical studies<sup>2</sup> demonstrated that it is a mixture of basic, water soluble antibiotics containing the aminocyclitol 2-deoxystreptamine and 2 additional amino sugars. Chromatographic separation of the gentamicin complex showed it to consist of 3 major components designated as C<sub>1</sub>, C<sub>2</sub> and C<sub>1a</sub>.<sup>3,4</sup> The gentamicin complex is used as the sulfate salt in various dosage forms including injectable and topical preparations, and is effective against a wide variety of gram-negative and gram-positive organisms.<sup>5</sup>

#### 1.2 Chemical Properties and Structure

Each of the three major components of the gentamicin complex contains five basic amino functions. As is typical of this class of antibiotics,<sup>6</sup> gentamicin sulfate is obtained as a hydrated amorphous solid without characteristic melting point, or UV absorption.

The elucidation of the structure and stereochemistry of the components of the gentamicin complex are described in publications by Cooper *et al.*<sup>7-11</sup> and Daniels.<sup>12</sup> The structural formulae, molecular weights and the nomenclature of the amino sugar units comprising the gentamicin complex are given in Figure 1; the common sugar unit has been named garosamine and the dissimilar 2,6-diamino sugars have been named purpurosamine A, B and C, corresponding to gentamicins C<sub>1</sub>, C<sub>2</sub>, and C<sub>1a</sub>, respectively.

A number of investigators have reported on minor components that are coproduced with gentamicin. In addition to gentamicin A and gentamicin B which were noted in the original paper by Weinstein *et al.*<sup>1</sup>, these minor components include gentamicins B<sub>1</sub>, X, C<sub>2a</sub>, and C<sub>2b</sub>. A summary of the methods used to isolate and separate these is given in a recent review.<sup>13</sup>



GENTAMICIN C <sub>1</sub>	R = R <sub>1</sub> = CH <sub>3</sub>	C <sub>21</sub> H <sub>43</sub> N <sub>5</sub> O <sub>7</sub> (M.W. 477.6)
GENTAMICIN C <sub>2</sub>	R = CH <sub>3</sub> ; R <sub>1</sub> = H	C <sub>20</sub> H <sub>41</sub> N <sub>5</sub> O <sub>7</sub> (M.W. 463.6)
GENTAMICIN C <sub>1a</sub>	R = R <sub>1</sub> = H	C <sub>19</sub> H <sub>39</sub> N <sub>5</sub> O <sub>7</sub> (M.W. 449.5)

Figure 1: Structural Formula of Gentamicin Complex.

### 1.3 Appearance, Color, Odor

Gentamicin sulfate is a white to buff colored, odorless, hygroscopic powder.

### 1.4 The USP Standard

The biological activity of bulk gentamicin sulfate is expressed in mcg gentamicin per mg gentamicin sulfate based on a potency of 1000 mcg per mg (dried basis) originally assigned to the master standard base. The current USP Standard of gentamicin sulfate has a potency of 650 mcg/mg on the dried basis and the minimum acceptance limit on potency for gentamicin sulfate bulk substance is 590 mcg/mg (dried basis). FDA certification also requires compliance with specifications for identity, pH, loss on drying, optical rotation and gentamicin C component ratios.<sup>14</sup>

## 2. Physical Properties

### 2.1 Infrared Spectrum

The infrared spectrum of a potassium bromide (KBr) pellet of Gentamicin Sulfate USP Reference Standard is given in Figure 2. It was obtained using a Perkin Elmer 180 grating spectrophotometer. The infrared band assignments are given below.<sup>15</sup> It should be noted that bands are not present which would permit differentiation from similar aminoglycoside antibiotics.

<u>Wavenumber (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3500-2500 (s, vbr)	OH, NH <sub>3</sub> <sup>+</sup> , NH <sub>2</sub> <sup>+</sup> stretch
1620 (m)	NH <sub>3</sub> <sup>+</sup> , NH <sub>2</sub> <sup>+</sup> symmetric bend
1525 (m)	NH <sub>3</sub> <sup>+</sup> , NH <sub>2</sub> <sup>+</sup> symmetric bend
1150-1000 (vs, br)	C-O, HSO <sub>4</sub> <sup>-</sup> stretch
610 (s)	SO <sub>2</sub> bend

Notation: w = weak, m = medium, s = strong, vs = very strong, br = broad, vbr = very broad.



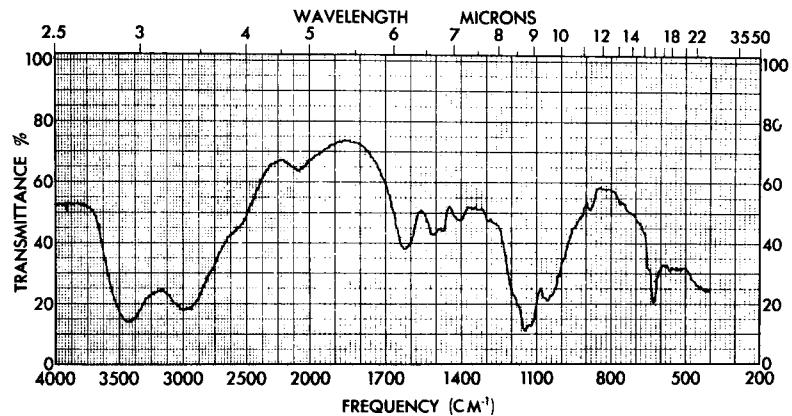


Figure 2: Infrared Spectrum (KBr pellet) of Gentamicin Sulfate USP Reference Standard.

## 2.2 Ultraviolet Spectrum

The gentamicin complex does not possess ultraviolet light absorbing properties; both the free base and sulfate show end absorption only.

## 2.3 Nuclear Magnetic Resonance Spectra

### 2.3.1 Proton Magnetic Resonance Spectrum

An 80 MHz proton NMR spectrum of a solution of Gentamicin Sulfate USP Reference Standard 15% w/v in D<sub>2</sub>O is given in Figure 3. It was obtained using a Varian CFT-20 spectrometer at ambient temperature and sodium 2,2-dimethyl, 2-silapentane-5-sulfonate (DSS) as the internal reference. The spectral assignments given below are in ppm ( $\delta$ ) downfield from DSS.<sup>15</sup>

<u>Protons</u>	<u>Chemical Shifts (<math>\delta</math>)</u>	<u>Multiplicity</u>	<u>Origin</u>
5'-CH( <u>CH</u> <sub>3</sub> )	1.30	doublets (J=7.5 Hz)	components of C <sub>1</sub> and C <sub>2</sub>
4''-CH <sub>3</sub>	1.35	singlet	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
2, 3', 4'CH <sub>2</sub>	1.75-2.5	broad	
5'-CH(CH <sub>3</sub> )NH <u>CH</u> <sub>3</sub>	2.75	singlet	C <sub>1</sub>
3''-NHCH <sub>3</sub>	2.95	singlet	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
3''-H	3.48	doublet (J=11.0 Hz)	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
5''-CH <sub>2</sub> O eq	4.0	multiplet	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
2''-H	4.25	doublet of doublet (J=11.0, 4.0 Hz)	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
1''-H	5.16	doublet (J=4.0 Hz)	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>
1-H	5.88	overlapping doublets	C <sub>1</sub> , C <sub>2</sub> , C <sub>1a</sub>

Additional discussion of NMR spectral assignments for gentamicin is given by Cooper *et al.*<sup>8,10,11</sup>

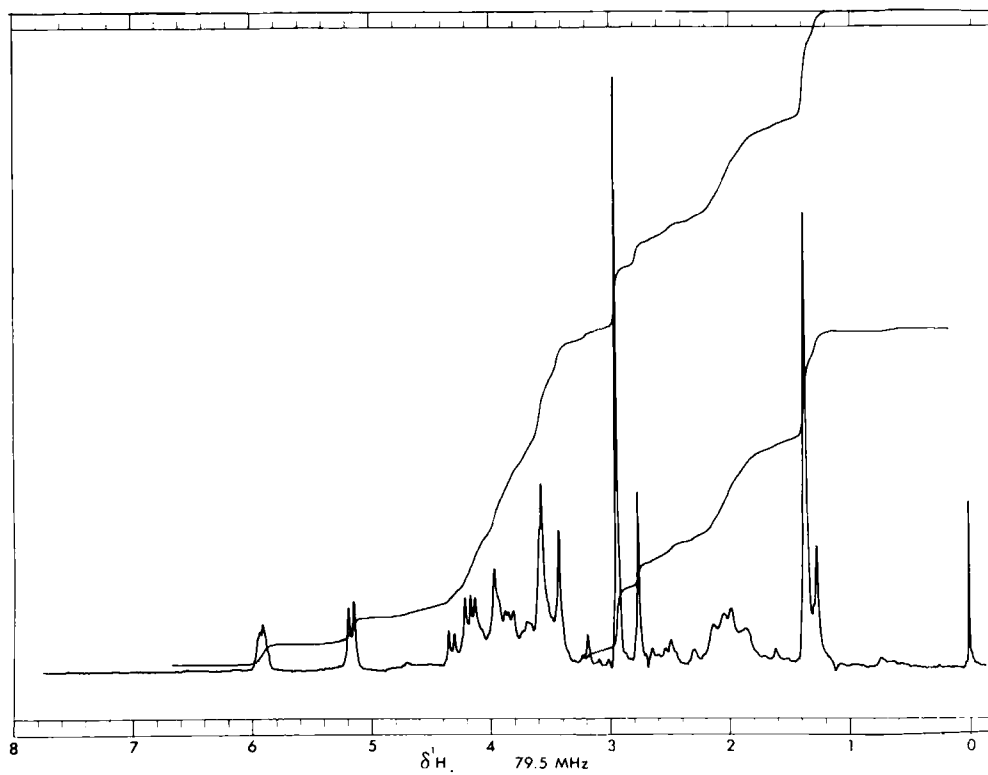


Figure 3: PMR Spectrum of Gentamicin Sulfate USP Reference Standard.

### 2.3.2 Carbon-13 Magnetic Resonance Spectrum

A carbon-13 NMR spectrum of a solution of Gentamicin Sulfate USP Reference Standard (80 mg/0.50 ml in D<sub>2</sub>O) is given in Figure 4. It was obtained using a Varian XL-100 spectrometer at ambient temperature and dioxane as the internal reference. The chemical shift assignments given in Table 1 are in ppm ( $\delta$ ) with reference to internal dioxane taken as 67.40 ppm down from external tetramethylsilane.<sup>15</sup>

A discussion of C-13 NMR spectral data of the gentamicin C components C<sub>1a</sub>, C<sub>2</sub>, and C<sub>1</sub> is given by Morton *et al.*<sup>16</sup>

### 2.4 Mass Spectrum

The mass spectrum of gentamicin free base, prepared by neutralization of Gentamicin Sulfate USP Reference Standard is given in Figures 5 and 5a. It was obtained using a Varian MAT CH-5 medium resolution single focusing spectrometer at a probe temperature of 170°C and a source temperature of 250°C. The mass assignments are given in Table 2.<sup>15</sup>

Additional discussion relating to the mass spectrometry of gentamicin is given by Cooper *et al.*<sup>8,11</sup>, Daniels *et al.*<sup>17,18</sup> and Parfitt *et al.*<sup>19</sup>

### 2.5 Thermal Properties (TGA, DSC)

#### 2.5.1 Thermogravimetric Analysis (TGA)

A thermogravimetric analysis curve was obtained for Gentamicin Sulfate USP Reference Standard (see Figure 6) using a DuPont Model 950 Thermogravimetric Analyzer equipped with a Model 900 Programmer-Recorder. The analysis was performed at a heating rate of 10°C/minute, under a nitrogen atmosphere.

The thermogravimetric analysis of the USP Reference Standard indicates loss of approximately 12% water from ambient to 125°C. Decomposition starts at 220°C and proceeds stepwise until 330°C; above 330°C additional decomposition occurs, yielding a final residue of about 30% which is attributable to the sulfate salt.<sup>15</sup>

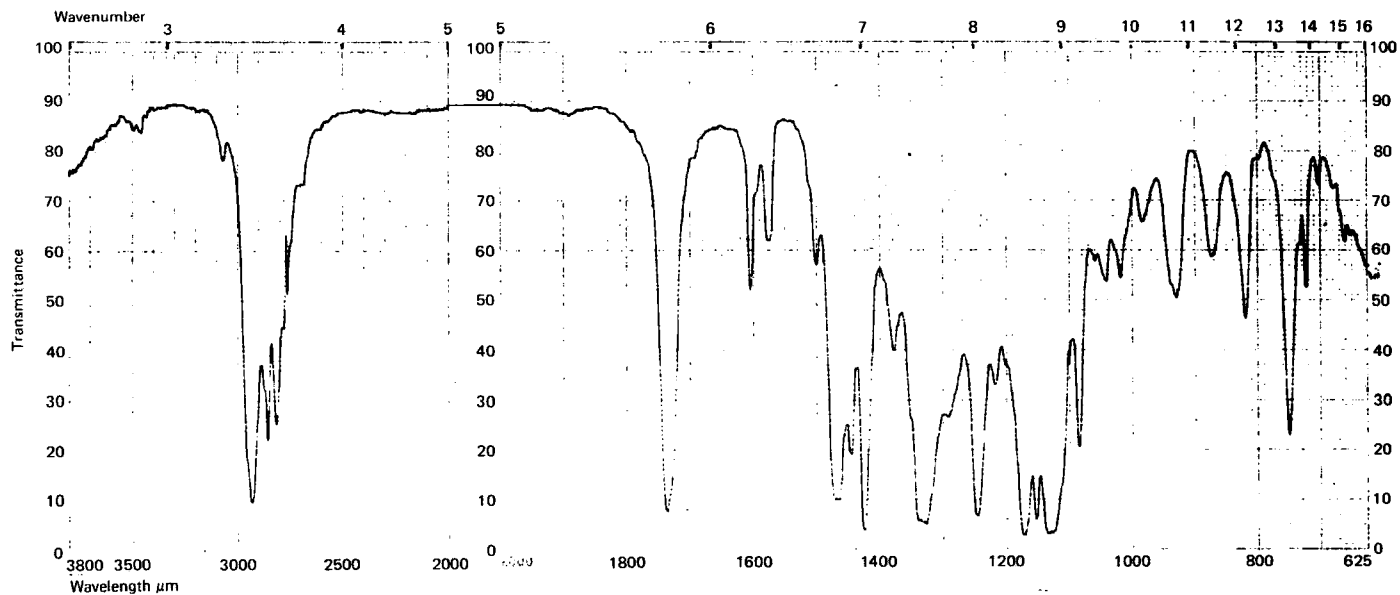


Figure 4: Carbon-13 NMR Spectrum of  
Gentamicin Sulfate USP Reference Standard.

TABLE 1

Carbon-13 chemical shift assignments of Gentamicin Sulfate USP Reference Standard in ppm ( $\delta$ ) with reference to internal dioxane taken as 67.40 ppm down from external tetramethylsilane (see Figure 4).

<u>Carbon</u>	<u>Chemical Shifts</u> <sup>1</sup>	
1	50.6	
2	28.5	
3	49.5	
4	76.7	
5	75.3	
6	84.4	
1'	95.4, 95.3, 95.0*	
2'	49.5	
3'	21.4	
4'	24.0(C <sub>2</sub> ,C <sub>1</sub> ), 26.3(C <sub>1a</sub> )	
5'	70.0, 69.6*	
5'-CH <sub>2</sub> NH <sub>2</sub>	43.5	C <sub>1a</sub>
5'-CH(CH <sub>3</sub> )NH <sub>2</sub>	50.4 (13.1)	C <sub>2</sub>
5'-CH(CH <sub>3</sub> )NH(CH <sub>3</sub> )	58.3 (10.1) (32.0)	C <sub>1</sub>
1"	102.0	
2"	67.1	
3"	64.3	
4"	70.8	
5"	68.7	
3"-NHCH <sub>3</sub>	35.4	
4"-CH <sub>3</sub>	21.8	

<sup>1</sup>The operating frequency of the spectrometer was 25.2 MHz (<sup>13</sup>C); 8K data points were acquired with a spectral width of 5500 Hz, a pulse width of 15.0  $\mu$ sec, yielding a flip angle of  $\sim 60^\circ$  and a repetition rate of 0.8 sec.

\*Multiplicity is due to the mixture of C components.

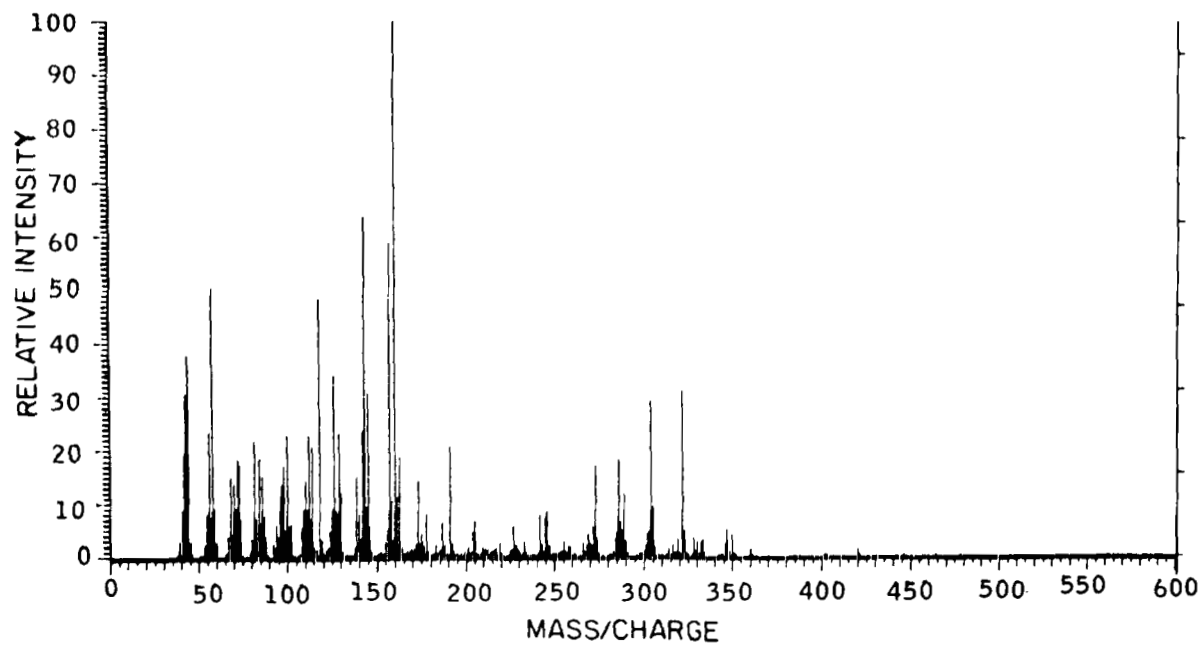


Figure 5: Mass Spectrum of Gentamicin Base  
(mass range 0 to 600).

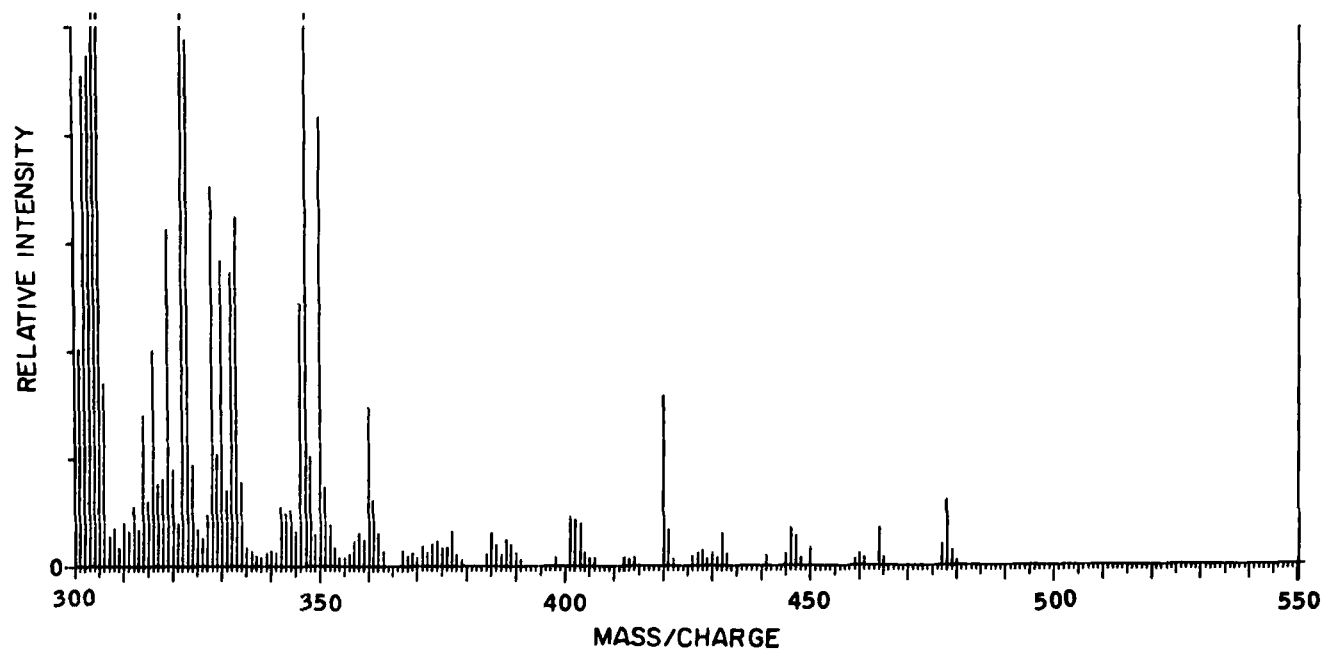


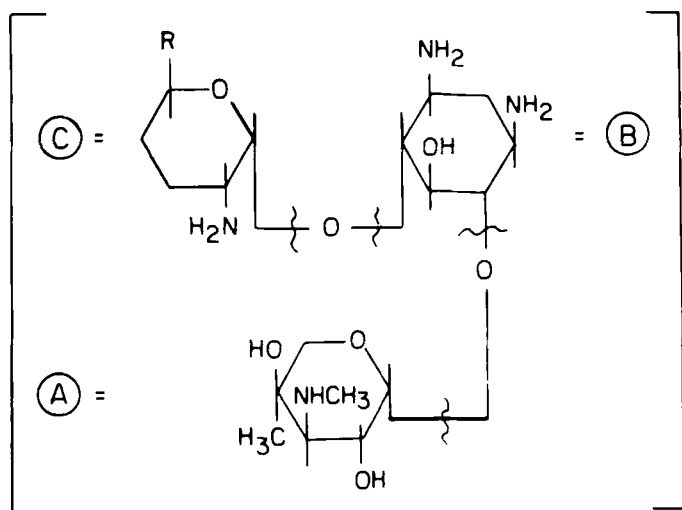
Figure 5a: Mass Spectrum of Gentamicin Base  
(mass range 300 to 550).



TABLE 2  
Mass Spectral Assignments of  
Gentamicin Base

Ions	<u>Masses (amu)</u>		
	<u>C<sub>1a</sub></u>	<u>C<sub>2</sub></u>	<u>C<sub>1</sub></u>
$(M+1)^+$	450	464	478
$(M-17)^+ (NH_3)$	432	446	460
$(A) - O - (B) - O^+ = CHOH$	350	350	350
$(C) - O - (B) - O^+ = CHOH$	319	333	347
$(A) - O - (B) - O^+H_2$	322	322	322
$[(A) - O - B]^+$	304	304	304
$HO (B) - O^+ = CHOH$	191	191	191
$(A)^+$	160	160	160
$(C)^+$	157	143	129
$[(B) OH]^+$	145	145	145

See the following page for the definition of A , B and C.

Table 2 (Continued)

$R = CH_2NH_2$   $C_{1a}$

$R = CH(CH_3)NH_2$   $C_2$

$R = CH(CH_3)NH(CH_3)$   $C_1$

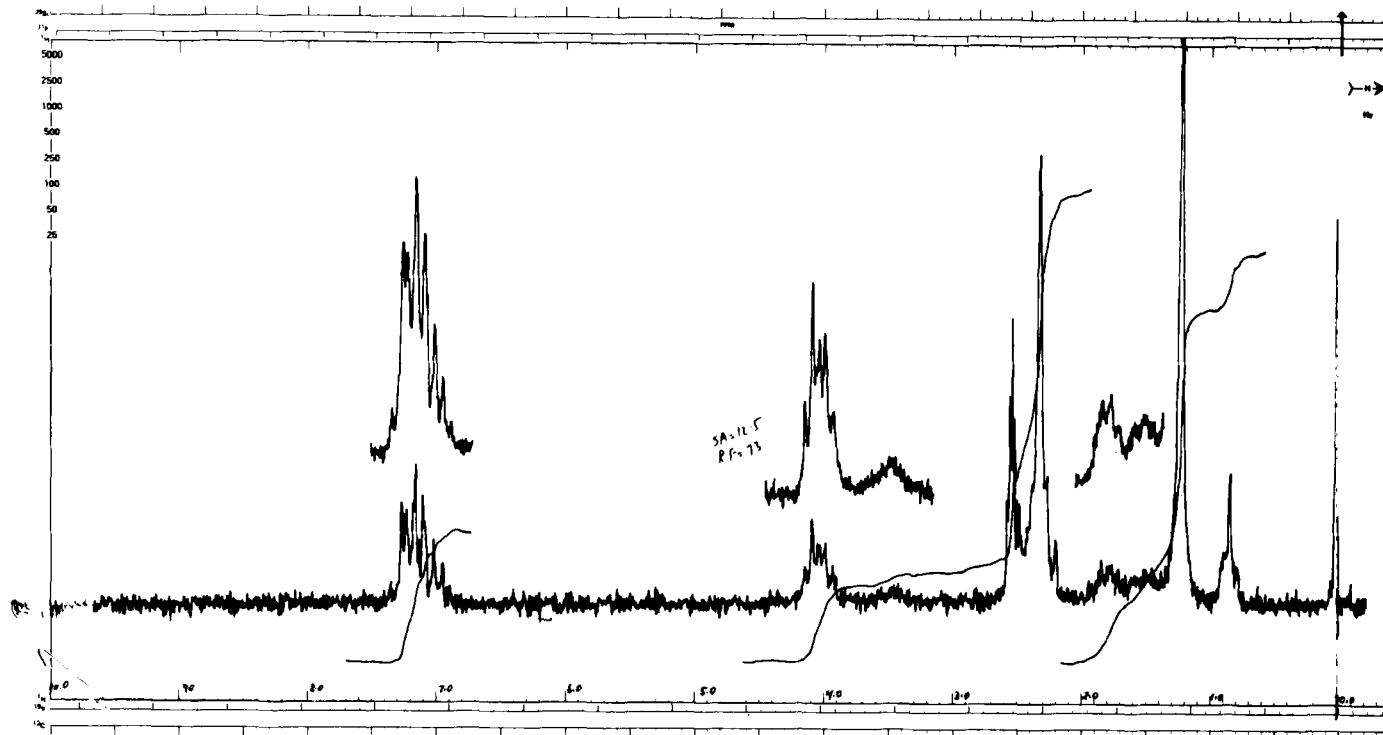


Figure 6: Thermogravimetric Analysis (TGA) Curve of Gentamicin Sulfate USP Reference Standard.

### 2.5.2 Differential Scanning Colorimetry (DSC)

A differential scanning calorimetry curve (see Figure 7) was obtained for Gentamicin Sulfate USP Reference Standard using a DuPont Model 990 Thermal Analyzer equipped with a Model 910 Cell Base. The scan was performed at a temperature program rate of  $10^{\circ}\text{C}/\text{minute}$ , under a nitrogen atmosphere against an empty aluminum sample pan.

The differential scanning calorimetry curve of the USP Reference Standard has a broad endothermic peak around  $75^{\circ}\text{C}$  due to loss of water and a large endotherm at  $250^{\circ}\text{C}$  corresponding to melting decomposition.<sup>15</sup>

### 2.6 Electrometric Titration Curve/Apparent pKa Value

Each of the three major gentamicin C components contains 5 basic amino groups. Because of their similar basic strength, the electrometric titration curve<sup>20</sup> (Figure 8) gives one titration "break" corresponding to five equivalents of acid consumed. An apparent pKa value (half neutralization) of 7.9 is derived from Figure 8. This curve was obtained with a Mettler automatic titration system (consisting of modules DV11, DK10 and DV103) and a Corning semimicro combination pH electrode. About 180 mg gentamicin base was dissolved in water and titrated with 0.5N hydrochloric acid. A pKa value of 8.2 for gentamicin was reported by Done<sup>21</sup> and also by Newton and Kluza.<sup>22</sup>

### 2.7 Optical Rotation

Allowable limits for the specific rotation of gentamicin sulfate are  $+107^{\circ}$  to  $+121^{\circ}$  as given in the Code of Federal Regulations (CFR)<sup>23</sup> as well as in the British Pharmacopoeia.<sup>24</sup> The CFR states that the measurement should be performed on a 1% aqueous solution at  $25^{\circ}\text{C}$ , while the British Pharmacopoeia states that a 10% aqueous solution should be measured at  $20^{\circ}\text{C}$ . The specific rotation of the USP Gentamicin Sulfate Reference Standard was found to be  $+115.9^{\circ}$  when measured as a 0.3% aqueous solution in a Bendix Series 1100 Polarimeter at  $26^{\circ}\text{C}$ .<sup>25</sup>

### 2.8 X-Ray Diffraction

X-ray powder diffraction studies<sup>26</sup> show that gentamicin sulfate is essentially an amorphous substance; no spectral bands were observed when the USP Reference Standard was run on a Philips APD-3500 utilizing Cu K $\alpha$  radiation ( $1.5418\text{\AA}$ ).

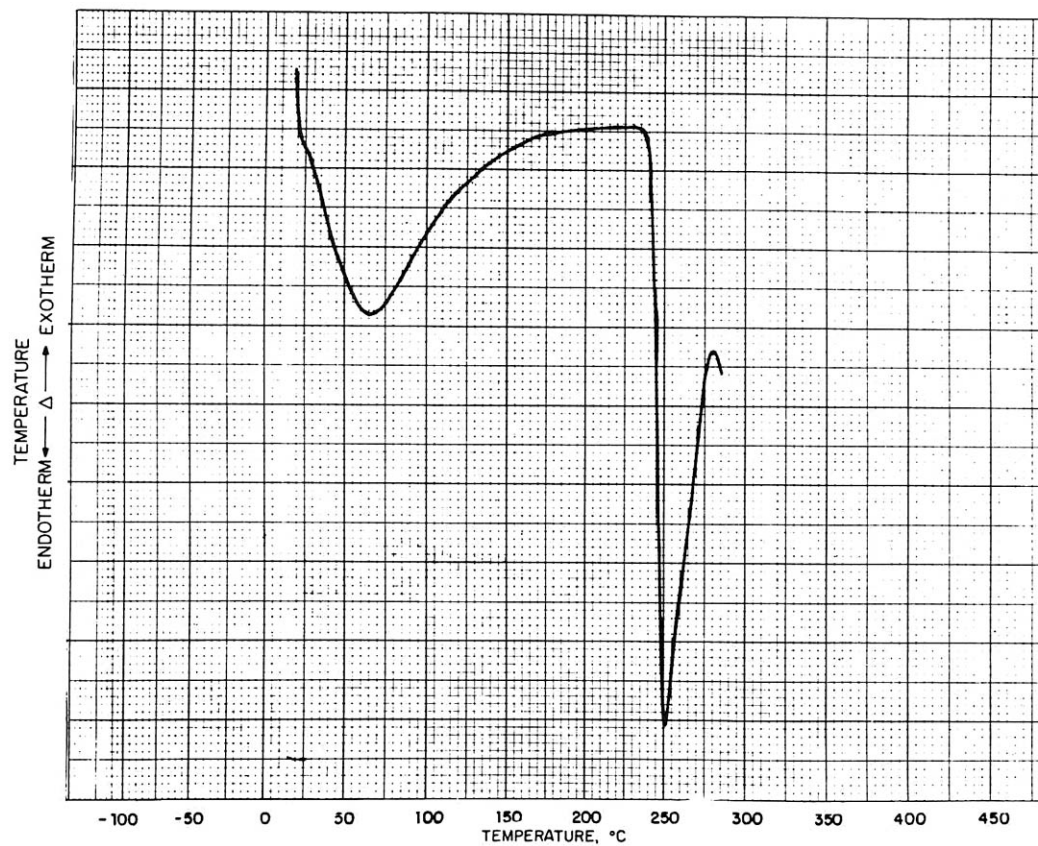


Figure 7: Differential Scanning Calorimetry (DSC) Curve of Gentamicin Sulfate USP Reference Standard.

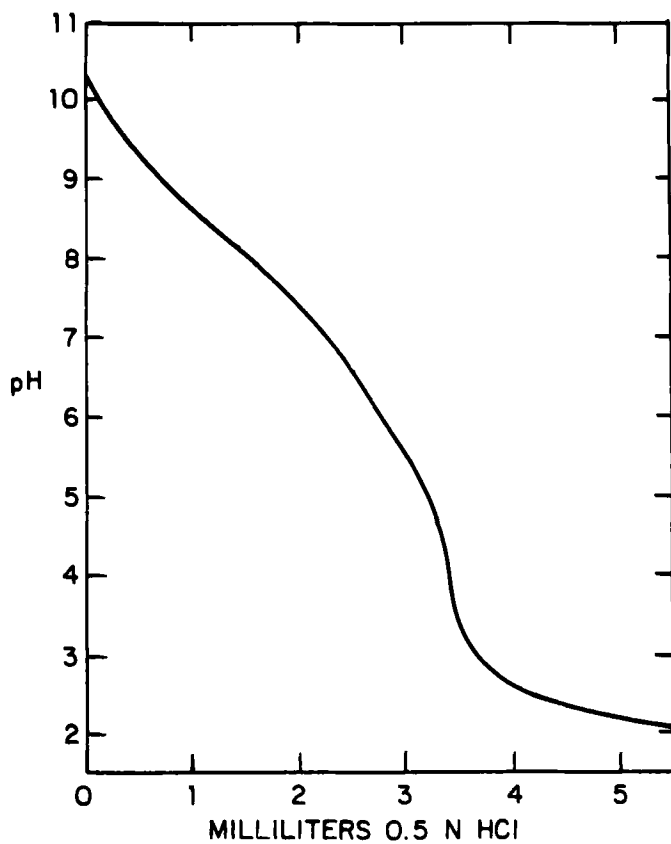


Figure 8: Electrometric Titration Curve of Gentamicin Base.

## 2.9 Solubility

Gentamicin sulfate is freely soluble in water, 0.1N hydrochloric acid, 0.1N sodium hydroxide (>1 g/ml in each of these aqueous media). It is insoluble in alcohol and most other organic solvents. As part of a comprehensive study of 51 antibiotic compounds Marsh et al.<sup>27</sup> reported the solubility of gentamicin sulfate in 26 solvents at room temperature. Some of these data are presented below:

<u>Solvent</u>	Solubility at 28+4°C
	<u>Gentamicin Sulfate</u> (mg/ml)
Ethylene Glycol	>20
Formamide	>20
Propylene Glycol	6.332
Chloroform	0.678
Methanol	0.200
Dimethyl Sulfoxide	0.072
Isopropanol	0.045
Acetone	0.042
Carbon Disulfide	0.028
Pyridine	0.028
Ethyl Acetate	0.025
Benzene	0.0
Carbon Tetrachloride	0.0
Isooctane	0.0
Diethyl Ether	0.0

Gentamicin base complex was found very soluble in water (>1 g/ml) and is more soluble than the sulfate salt in a number<sup>25</sup> of organic solvents. Some of these data are tabulated below:

<u>Solvent</u>	Solubility at 25+1°C
	<u>Gentamicin Base</u> (mg/ml)
Methanol	>25
n-Butanol	>25
Ethanol	>25
Chloroform	>25
Acetone	>25
2-Butanone	2.6
Toluene	2.4
Ethyl Acetate	2.1
Cyclohexane	0.2

## 2.10 Countercurrent Distribution

In 1977, Byrne *et al.*<sup>28</sup> reported on the separation of the gentamicin C complex into five components by Craig distribution. In addition to the three major components  $C_1$ ,  $C_2$ , and  $C_{1a}$ , these workers separated two additional components,  $C_{2a}$  and  $C_{2b}$ . Gentamicin  $C_{2a}$  was identified as the 6'-C-epimer of gentamicin  $C_2$ , while gentamicin  $C_{2b}$  was identified as 6'-N-methylgentamicin  $C_{1a}$ . The separations were carried out in a 1020-cell automatic Craig distribution apparatus of 10 ml fixed lower phase volume, using a chloroform:methanol:17% ammonia (2:1:1) solvent system.

## 3. Biosynthesis

The biosynthesis of aminocyclitol antibiotics, including gentamicin, is discussed in a recent comprehensive review.<sup>29</sup> Glucose has been shown to provide the skeletons of all subunits of the antibiotics so far studied; however, details of the steps involved are still unknown in almost all cases. Of the deoxystreptamine-containing antibiotics, the bulk of the effort has been directed toward the biosynthesis of neomycins.

Gentamicins differ from the neomycins, kanamycins, and paromomycins in that they contain both C-methyl and N-methyl substituents; most studies on gentamicins have been aimed at determining the source of the methyl groups. Studies carried out by Lee *et al.*<sup>30</sup> indicate a high efficiency of L-methionine incorporation into gentamicins. Labelling experiments using  $^{13}\text{C}$ -methyl methionine and  $^3\text{H}$ -methyl methionine have shown that all of the methyl groups in gentamicin are derived from methionine.<sup>31</sup> Additional work by Lee *et al.*<sup>32</sup> shows that when  $^{13}\text{C}$ -methyl-methionine was added at the onset of biosynthesis of the gentamicin components, incorporation of label into the minor components preceded incorporation into the major components. Degradation occurred when  $^{14}\text{C}$ -methyl gentamicin major components were added to the gentamicin-producing culture medium and shaken.

## 4. Isolation and Purification Processes

In 1963 Rosselet<sup>33</sup> and co-workers first reported on the isolation of the gentamicin complex using ion-exchange chromatography. Various ion-exchange procedures continue to be used extensively for the separation and purification of gentamicin on a preparative scale. A commonly used procedure is to adjust the whole broth to pH 2 with sulfuric acid, followed by



filtration. After adjustment to pH 7, the neutralized filtrate is passed through an IRC-50 resin column in the ammonium cycle, and the antibiotic is then eluted with 2N aqueous ammonia. The gentamicin C complex may be isolated from co-produced minor components using a Dowex 1X2 column (OH-form).<sup>5</sup>

## 5. Drug Metabolism and Pharmacokinetics

Gentamicin shares with many other aminoglycoside antibiotics the important property of being stable in biological systems. When administered to man or animals, the major portion is excreted in the urine by glomerular filtration.<sup>34</sup> Gentamicin is not absorbed in appreciable amounts from the intact gastrointestinal tract.

After intramuscular administration, peak serum concentrations usually occur between 30 and 60 minutes and serum levels are measurable for six to eight hours. When gentamicin is administered by intravenous infusion over a two-hour period, the serum concentrations are similar to those obtained by intramuscular administration. Protein binding studies have indicated that the degree of gentamicin binding is low, between 0 and 30%.<sup>35</sup>

## 6. Stability

Gentamicin sulfate powder is very stable when stored in tightly closed containers at room temperature. Gentamicin sulfate is stable for at least five years with respect to potency, specific rotation and pH.

Gentamicin was also stable in boiling aqueous buffers of pH 2 to 14.<sup>36</sup> It is particularly resistant to attack by alkali, and has been refluxed in 2N sodium hydroxide for 72 hours with no apparent loss in activity.<sup>37</sup> More recent studies on gentamicin confirm its excellent stability in moderately acid to strongly basic aqueous media. Under highly stressed conditions (heating in 1N sulfuric acid for 5 days at 60°C), approximately a 30% loss in potency was found.<sup>38</sup> Gentamicin sulfate was also shown to be stable in infusion solutions<sup>39</sup> and in artificial tear solutions.<sup>40</sup>

Gentamicin sulfate exhibits excellent stability in various pharmaceutical dosage forms. In parenteral solutions and topical ointments it has been shown to be stable for at least five years under normal storage conditions (2° to 30°C).

## 7. Methods of Analysis

### 7.1 Identification

Gentamicin is conveniently identified by thin-layer chromatography (TLC). Gentamicin is resolved into its 3 components and also can be separated from most other related antibiotics using TLC. Refer to the discussion in section 8.2 and especially Wilson *et al.*<sup>41</sup> and Pauncz<sup>42</sup> for related discussion.

In a typical TLC method about 50 to 100 µg of gentamicin sulfate is applied to a silica gel TLC plate and developed using the lower phase of a mixture of equal volumes of chloroform, methanol and concentrated aqueous ammonia.<sup>41</sup> The spots are typically visualized with ninhydrin reagent or with iodine vapors. Results are compared with those obtained from a similarly chromatographed reference solution.

Paper chromatography is also useful for identification (see section 8.1). The British Pharmacopoeia 1973, p. 216 describes a method where the solvent system chloroform:methanol:concentrated aqueous ammonia:water (10:5:3:2) is used along with ninhydrin spray detection.

The BP also describes a method where a UV spectrum is obtained after treatment with sulfuric acid. No maximum is obtained for gentamicin, which distinguishes it from kanamycin, neomycin and paromomycin.

The Code of Federal Regulations (444.20) describes an infrared spectrophotometric technique using a KBr disc. The infrared spectrum of gentamicin sulfate, however, is very similar to that of other aminoglycoside antibiotics and is therefore of limited value as an identification test.

### 7.2 Determination of Sulfate Content

As described in section 1.2, gentamicin sulfate is composed of three major components. Since each component has 5 basic nitrogens, 5 equivalents of sulfuric acid are required per mole of gentamicin base. The limits for sulfate content given in the British Pharmacopoeia 1973 are 31.0 to 34.0% (anhydrous basis).

The gravimetric procedure described in the BP<sup>43</sup> involves precipitation of barium sulfate by the addition of hydrochloric acid and barium chloride to an aqueous solution

of the antibiotic, followed by washing, igniting and weighing the residue. Each gram of residue is equivalent to 0.4116 gram of sulfate.

### 7.3 Loss on Drying and Moisture Content

Gentamicin sulfate is an amorphous, hygroscopic powder which typically contains 10 to 15% water. The U.S. Government Code of Federal Regulations (CFR) allows a maximum of 18% loss on drying.<sup>44</sup> In the CFR method<sup>45</sup> the gentamicin sulfate sample is heated at a temperature of 110°C for 3 hr in a vacuum ( $\leq 5$ mm mercury). The British Pharmacopoeia specification for water content is 15%<sup>24</sup> using Karl Fischer titration and electronic endpoint detection.<sup>46</sup>

It has been shown that loss on drying results and Karl Fischer titration results are in good agreement.<sup>47</sup>

### 7.4 Determination of Component Ratios

A number of methods have been used for the determination of gentamicin C component ratios. Brief descriptions of nine of these methods are presented in this section.

7.4.1 For U.S. certification, all batches of gentamicin sulfate must conform to the following requirements for component ratios described in the Code of Federal Regulations:

$C_1$ :	Not less than 25% nor more than 50%
$C_1^a$ :	Not less than 15% nor more than 40%
$C_2$ :	Not less than 20% nor more than 50%

The official method given in 21 CFR 444.20 a(b)(8) is based on a paper chromatographic procedure reported by Kantor and Selzer.<sup>48</sup> In this method, two identical sample chromatograms are developed in the lower phase of chloroform-methanol-17% ammonium hydroxide (2:1:1). One chromatogram is sprayed with ninhydrin reagent to locate the positions of the C components  $C_1^a$ ,  $C_2$  and  $C_1$ , which have approximate  $R_f$  values of 0.35, 0.50<sup>2</sup> and 0.75 respectively. The spots in this strip are used to locate the corresponding zones in the second strip. The zones are cut out, eluted with pH 8.0 0.1M phosphate buffer, and assayed using the CFR microbiological agar diffusion assay.

7.4.2 Wagman et al.<sup>49</sup> reported a differential chromatographic bioassay for the gentamicin complex. The three gentamicins are separated using the same paper chromatography

system described in section 7.4.1. After chromatographic development, the paper strips are dried and plated against Staphylococcus aureus ATCC 6538P and the zones of inhibition are quantitated using standard zone response curves.

7.4.3 In another modification using the same paper chromatography system described in Section 7.4.1, the developed papers are sprayed lightly with dilute 2,4,6-trinitrobenzenesulfonic acid (TNBSA) to detect the gentamicin C components, which appear as yellow zones. The zones are cut out, additional TNBSA in a pH 9.4 buffer is added and the chromophore is allowed to develop at 30°C for one hour. The amount of each gentamicin C component is quantitated by comparison of the absorbances obtained at 420 nm with those obtained from a similarly treated chromatogram of the reference standard.<sup>25</sup>

7.4.4 Wagman *et al.*<sup>50</sup> reported a differential ninhydrin paper chromatographic assay for the gentamicin complex. After development, the paper strips are dried and sprayed with ninhydrin reagent. The color is developed with heat and the color intensities are read on an integrating scanner. The proportions of the three gentamicin C components in the sample are calculated by comparison to standards of the three individual C components similarly treated.

7.4.5 Anhalt *et al.*<sup>51</sup> reported a high pressure liquid chromatographic method for gentamicin C component determination. In this method a reversed phase LC column separates the three gentamicin C components by paired-ion chromatography. The separated components are derivatized with o-phthalaldehyde to give fluorescent products. Results from this procedure compare favorably with assay results by the CFR microbiological agar diffusion method. See Section 8.5 for an expanded discussion of this HPLC technique.

7.4.6 Kabasakalian *et al.*<sup>52</sup> reported a method for the determination of the gentamicin components in fermentation broth by in-situ fluorimetric measurements of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD) derivatives. In this method, fermentation broth samples are acidified, centrifuged, adjusted to pH 12, spotted on TLC plates and developed. The dried plates are dipped in methanolic NBD chloride, heated, cooled and rechromatographed in methanol. The fluorescent spots are scanned and integrated using a densitometer. This method provides a rapid means of following changes in component ratios during the course of the fermentation.

7.4.7 Calam et al.<sup>53</sup> reported a method for control and monitoring the properties of the three gentamicin C components by  $^1\text{H}$  nuclear magnetic resonance spectroscopy. This method involves measurement of the peak heights of signals for N-methyl and C-methyl groups present in all three components and of those present in  $\text{C}_1$  and  $\text{C}_2$  only. The peak height ratios are calculated. The results are used to control and monitor composition within certain limits and not to determine the actual % composition of each component. The limits and the method appear in the gentamicin sulphate monograph of the British Pharmacopoeia 1973, Addendum 1975.

The gentamicin C components have been monitored using  $^{13}\text{C}$  NMR by 24 hour accumulation of spectra.<sup>54</sup>

7.4.8 Thomas and Tappin<sup>55</sup> reported an ion-exchange method with direct optical rotation measurement that is useful for examining C component distribution in gentamicin sulfate. In this method, 80 mg samples of gentamicin sulfate are dissolved in 0.5 ml 2 M sodium chloride and added to the top of a column (0.9 x 15 cm) filled with cellulose phosphate P-11 ion-exchange material. A gradient mixer delivers sodium chloride solution in increasing molarity to the column. The column eluate is monitored by a polarimeter with a flow-through microcell. The output is plotted on a potentiometer recorder. The peak areas are determined with a planimeter and expressed as percent of the total area.

See Thomas<sup>56</sup> for a comparison of results obtained by the CFR microbiological method, the  $^1\text{H}$  NMR method, and the above ion-exchange method.

7.4.9 Wilson et al.<sup>57</sup> reported a gentamicin C component assay method using thin-layer chromatography followed by direct densitometry. Gentamicin sulfate is spotted on silica gel TLC plates followed by development in the lower phase of chloroform-methanol-concentrated ammonium hydroxide (1:1:1). After drying, the plates are sprayed with ninhydrin reagent yielding magenta spots on a white background. The spots are examined by direct densitometry and quantitated with a digital integrator. The authors claim that this method is faster and offers the same precision as microbiological methods.

## 7.5 Microbiological Assay

In 1963 Oden *et al.*<sup>58</sup> described a standard curve disc-plate agar diffusion assay using Staphylococcus aureus ATCC 6538P as the test organism for the analysis of gentamicin raw materials. In this paper a standard curve cylinder-plate assay utilizing Bacillus subtilis ATCC 6633 was also reported for the determination of gentamicin in serum samples. (refer to section 10.1) Factors affecting the assay results using these assay procedures, such as the effect of salts in the agar media, are described.

The current official microbiological assay procedure described in the U.S. Code of Federal Regulations (CFR)<sup>59</sup> for the substance and dosage forms is a cylinder plate assay using Staphylococcus epidermidis ATCC 2228 as the test organism. The British Pharmacopoeia utilizes<sup>60</sup> a cylinder plate assay and Bacillus pumilus NCTC 8241 as the test organism. Detailed procedures for carrying out the assays are given in the compendia.

The minimum potency required by both the CFR and BP for acceptance of bulk commercial gentamicin sulfate is 590 mcg per mg on the dried (anhydrous) basis.

## 8. Chromatographic Analysis

### 8.1 Paper Chromatography

Gentamicin can be separated into its three components ( $C_{1a}$ ,  $C_2$ ,  $C_1$ ) by descending paper chromatography using the solvent system chloroform:methanol:17% aqueous ammonia (2:1:1, lower phase).<sup>4</sup> This is a modification of the system given by Ikekawa *et al.*<sup>61</sup> The approximate  $R_f$  values reported for the three gentamicin C components<sup>62f</sup> are :

<u>Component</u>	<u><math>R_f</math></u>
$C_{1a}$	0.21
$C_2$	0.40
$C_1$	0.67

Other paper chromatography systems have been reported but provide little or no separation of the three gentamicin C components.<sup>62-65</sup>

Gentamicin can be detected by spraying with ninhydrin reagent<sup>4</sup> (0.25% ninhydrin in pyridine-acetone 1:1) followed by heating at 105° for several minutes. The spots produced are purple-blue in color against a white background. Ninhydrin reagent prepared by dissolving 1 gram of ninhydrin and 0.1 gram of cadmium acetate in a solution of 3 ml water, 1.5 ml glacial acetic acid and 100 ml of *n*-propanol has also been used.<sup>48,66</sup> A bioautography method<sup>4</sup> may also be used where the paper strip is placed on agar seeded with Staphylococcus aureus ATCC 6538P. The  $R_f$  values of the resulting zones of inhibition are the same as the spots produced with ninhydrin detection.

Table 3 is a summary of paper chromatography systems for gentamicin and contains references to qualitative and quantitative methods. An expanded discussion of quantitative methods is given in Section 7.4.

## 8.2 Thin-Layer Chromatography

Thin-layer chromatography is an effective means of identifying and separating the components of the gentamicin complex. Table 5 gives a list of TLC systems that have been used for gentamicin. Reviews concerning TLC of gentamicin and related antibiotics are available.<sup>62,65,67,70</sup>

TLC is very useful for separating gentamicin from other related aminoglycoside antibiotics. Ito *et al.*<sup>68</sup> lists  $R_f$  values for gentamicin and 14 other basic water soluble antibiotics using solvent system C in Table 5. Pauncz<sup>42</sup> separated gentamicin from several other deoxystreptamine containing antibiotics and their decomposition products using solvent system E. This system did not, however, separate gentamicin into its three components.

Kabasakalian *et al.*<sup>52</sup> reported a quantitative TLC method for the gentamicin complex using fluorimetric detection. See Section 7.4 for an expanded discussion of this method.

Table 3

Paper Chromatography Systems for Gentamicin

<u>Method Type</u>	<u>Paper</u>	<u>Solvent</u> See Table 4	<u>Detection</u> See Table 4	<u>R<sub>f</sub> Values</u> <u>C<sub>1a</sub>, C<sub>2</sub>, C<sub>1</sub></u>	<u>Reference</u>
Qualitative	Whatman No. 1	A	1	0.59*	1
Qualitative	Whatman No. 1	B	1	0.26*	1
Qualitative	Whatman No. 1	C	1	0.10*	1
Qualitative	Whatman No. 1	D	1	0.30*	1
Qualitative	Whatman No. 1	E	1,2	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	4
Quantitative	Whatman No. 1	E	1	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	49
Quantitative	Whatman No. 4	E	3	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	48
Quantitative	S & S No. 589	E	2	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	50

\*Gentamicins C<sub>1a</sub>, C<sub>2</sub>, and C<sub>1</sub> are not separated.



Table 4

Paper Chromatography Solvent Systems for Gentamicin

- A. Methanol:water (4:1) + 3% sodium chloride vs. paper buffered with 0.95 M sodium sulfate + 0.05 M sodium bisulfate.
- B. Propanol:pyridine:acetic acid:water (15:10:3:12).
- C. Propanol:water:acetic acid (50:40:5).
- D. Aqueous phenol, 80%.
- E. Lower phase of chloroform:methanol:17% ammonium hydroxide (2:1:1)

Paper Chromatography Detection Methods for Gentamicin

- 1. Bioautography vs. Staphylococcus aureus ATCC 6538P.
- 2. Spray with 0.25% ninhydrin in pyridine:acetone (1:1). Heat at 105°C for several minutes giving purple to blue spots.
- 3. Spray with Modified Barrolier reagent. Add 3 ml water and 1.5 ml glacial acetic acid to 1 g ninhydrin and 0.1 g cadmium acetate and shake. Add to 100 ml n-propanol and shake until solution is complete.

Table 5Thin-Layer Chromatography Systems for Gentamicin

<u>Plate Medium</u> (see below)	<u>Solvent</u> (see below)	<u>Detection</u> (see below)	<u>R<sub>f</sub> Values</u> <u>C<sub>1a</sub>, C<sub>2</sub>, C<sub>1</sub></u>	<u>Reference</u>
a	A	1	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	41
b	B	1,2,3	C <sub>1a</sub> < C <sub>2</sub> < C <sub>1</sub>	4
c	C	1	0.20, 0.28, 0.35	68
b	D	1,2	0.69, 0.76, 0.71	69
d	E	1	0.05*	42

\*Gentamicins C<sub>1a</sub>, C<sub>2</sub>, and C<sub>1</sub> are not separated.

Plate Medium

- a. Silica Gel 60, 0.25 mm thickness, E.M. Laboratories
- b. Silica Gel G
- c. MN cellulose powder 300, 250 micron thickness
- d. Dowex 50 x 8 ion-exchange resin-coated plate

Table 5 (Continued)

Solvent System

- A. Lower phase of chloroform-methanol-concentrated ammonium hydroxide (1:1:1).
- B. Lower phase of chloroform-methanol-17% ammonium hydroxide (2:1:1).
- C. n-Propanol-pyridine-glacial acetic acid-water (15:10:3:12).
- D. Chloroform-methanol-concentrated ammonium hydroxide-water (1:4:2:1).
- E. 1.5 M Sodium acetate (pH 8.5) containing 1.0 M sodium chloride and 10% tert-butanol.

Detection Method

- 1. Ninhydrin reagent spray (see references for details of reagent preparation).
- 2. Bioautography vs. Staphylococcus aureus ATCC 6538P.
- 3. Starch-potassium iodide reagent spray.

### 8.3 Ion-Exchange Chromatography

Ion-exchange chromatography has been used extensively for the preparative separation of gentamicin components. Gentamicins may be isolated from fermentation media by passage over ion-exchange resins; 20-50 mesh Amberlite IRC-50, sodium form<sup>33</sup>, Amberlite IRC-50, ammonium form<sup>69</sup>, and KB-2-7p<sup>71</sup> resins have been used. The gentamicin C components may be separated from the minor A, B, and X components on a strongly basic resin, Dowex 1X2 with deionized water as eluent<sup>69,72</sup>. Basic ion exchange resins such as Amberlite IRA-400 (OH-) are used to convert gentamicin sulfates to the corresponding free bases.<sup>33</sup>

An ion-exchange separation has been suggested as a quantitative measure of C component ratios and content in gentamicin samples.<sup>56</sup> See Section 7.4 for additional discussion of this paper.

### 8.4 Gas Chromatography

Since gentamicin has relatively low volatility, all gas chromatographic analyses involve derivatization. Cunningham and Matsen<sup>73</sup> hydrolyzed serum gentamicin with 6 M HCL and analyzed the resulting 2-deoxystreptamine as its trifluoroacetyl derivative on a 3% OV-101 column at 150°C with flame ionization detection. Mayhew and Gorbach<sup>74,75</sup> determined serum gentamicin by a two step derivatization with N-trimethylsilylimidazole and N-heptafluorobutyrylimidazole; quantitative measurements were made by means of an electron capture detector following chromatography on 3% OV-101 supported on Chromosorb W AW DMCS.

### 8.5 High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) has been used as an assay method both for total gentamicins and for individual components. Samples which have been analyzed include serum and urine specimens as well as the bulk drug material.

A major obstacle to gentamicin HPLC assays has been the problem of detection. Gentamicin exhibits no significant UV bands above 190 nm and has no native fluorescence. In addition, assay levels are generally too low for the use of the refractive index detector. Hence, the several methods which have been developed involve derivatization of the drug.

Peng, et al.<sup>76</sup> detected gentamicin as its dansyl derivative. Following deproteinization of serum, gentamicin was derivatized with dansyl chloride and extracted into ethyl acetate. The sample was then chromatographed on a microparticulate reversed phase column by using an aqueous acetonitrile eluent and fluorescence detection.

Maitra et al.<sup>77,78</sup> separated gentamicin from serum by means of a silicic acid column. The drug was then reacted with o-phthalaldehyde, chromatographed on a  $\mu$ Bondapak C<sub>18</sub> column with a methanol:water (79:21) mobile phase containing tripotassium EDTA and detected by fluorescence readout.

Continuous-flow post-column derivatization of gentamicin with o-phthalaldehyde was performed by Anhalt.<sup>79,80</sup> The drug was determined by fluorescence readout after separation on a  $\mu$ Bondapak C<sub>18</sub> column with a mobile phase of methanol:water (3:97) with 0.2 M Na<sub>2</sub>SO<sub>4</sub>, 0.02 M sodium pentanesulfonate and 0.1% (v/v) acetic acid. Anhalt, et al.<sup>51</sup> compared this method to a normal phase separation on a Partisil (30 cm x 3.9 mm i.d.) column with a diethylamine:methanol:water (0.5:40:60) mobile phase and refractive index detection.

An elaboration on the method of Anhalt includes the use of netilmicin as internal standard and resolves minor impurities in the bulk drug substance.<sup>81</sup> Separations are obtained on an E.M. Merck RP-8 column, mobile phase of 0.2 M sodium sulfate, 0.02 M sodium pentanesulfonate, 0.1% (v/v) acetic acid, and post column derivatization with o-phthalaldehyde. This method gives a linear response to gentamicin over a wide concentration range, up to 0.15 mg/ml for each component. It is specific, accurate and precise, and has been used effectively to determine the gentamicin content of bulk samples as well as finished products.

## 9. Electrophoresis

Electrophoresis has been used to separate gentamicin from other antibiotic drugs in serum and in urine. Various systems which have been used for these separations are given in Table 6. Whiteley and co-workers<sup>82</sup> have used an electrophoretic separation to demonstrate an in-vitro interaction between gentamicin and cephalixin.

Table 6

Conditions for Electrophoresis of Gentamicin

<u>Medium</u>	<u>Buffer</u>	<u>Detection Method</u>	<u>Gentamicin Mobility Relative to Neomycin</u>	<u>Reference</u>
Filter Paper (Whatman No. 4)	1 <u>M</u> formic acid 0.005 <u>M</u> p-toluenesulfonic acid in n-PrOH:H <sub>2</sub> O (5:95) pH 1.8	Microbiological	0.95	83
Filter Paper (Whatman No. 4)	0.8 <u>M</u> formic acid 0.005 <u>M</u> p-toluenesulfonic acid in n-PrOH:H <sub>2</sub> O (5:95) pH 1.9	Microbiological	1	83
Filter Paper (Whatman No. 4)	0.8 <u>M</u> formic acid 0.005 <u>M</u> p-toluenesulfonic acid in H <sub>2</sub> O pH 1.9	Microbiological	1	83
Filter Paper (Whatman No. 4)	0.2 <u>M</u> NH <sub>4</sub> OH 0.005 <u>M</u> NaOH 0.01 <u>M</u> p-toluenesulfonic acid in n-PrOH:H <sub>2</sub> O (1:9) pH 10.8	Microbiological	1.74	83
Filter Paper (Whatman No. 4)	0.2 <u>M</u> NH <sub>4</sub> OH, 0.0025 <u>M</u> NaOH, in H <sub>2</sub> O pH 11.5	Microbiological	1.43	83
Filter Paper (Whatman No. 4)	0.05 <u>M</u> NaOH in H <sub>2</sub> O pH 12.2	Microbiological	1.02	83

Table 6 (Continued)

Conditions for Electrophoresis of Gentamicin

<u>Medium</u>	<u>Buffer</u>	<u>Detection Method</u>	<u>Gentamicin Mobility Relative to Neomycin</u>	<u>Reference</u>
Filter Paper (Whatman 3 MM)	formic acid:acetic acid: water (6:24:170)	0.25% ninhydrin, 0.01% isatin, 1% lutidine in acetone	1	84
0.9% agarose in pH 5.6 buffer	Tris-(hydroxymethyl)- aminomethane, maleic acid in H <sub>2</sub> O pH 5.6	Bio-autographical	0.85	85
Filter Paper (Whatman No. 1)	0.05 M NH <sub>4</sub> OAc, 0.05 M NH <sub>4</sub> OH in H <sub>2</sub> O pH 9.4	Ninhydrin	n.a.	82

## 10. Determination in Body Fluid

Assays for gentamicin in body fluids have been performed using a number of methods; these include microbiological assays, immunoassays, radioenzyme assays and high pressure liquid chromatographic assays.

### 10.1 Microbiological Assay

The microbiological assay of gentamicin is an agar diffusion assay based upon a comparison between the growth inhibition zones produced by the test sample and those produced by gentamicin standard of known potencies diluted in appropriate body fluid. Oden *et al.*<sup>58</sup> described a standard curve cylinder-plate assay utilizing Bacillus subtilis ATCC 6633 as the test organism with a sensitivity of 0.05 mcg/ml. Alcid and Seligman<sup>86</sup> reported the use of a multiple antibiotic-resistant strain of Staphylococcus epidermidis ATCC 27626 as the test organism. Gentamicin concentrations are estimated using this test organism in the presence of other antibiotics without the use of enzymes, radioactive material, or elaborate equipment and techniques.

### 10.2 Fluoroimmunoassay

A fluorometric immunoassay for gentamicin in serum was developed by Watson and co-workers<sup>87</sup>, who have prepared a fluorescein isothiocyanate derivative of the drug. The principle upon which the assay is based is that a complex of the derivatized gentamicin with antibody would scatter incident polarized light more than the smaller uncomplexed derivative molecule. The degree of antibody binding thus can be determined from changes in the fluorescence intensity.

### 10.3 Radioimmunoassay

Radioimmunoassay for gentamicin is based on the binding of the antibiotic by an appropriate antibody. By adding both antibody and radio-labelled drug to a sample containing gentamicin, one establishes an equilibrium between the bound and free labelled and unlabelled gentamicin molecules. Following separation of the bound and free fractions, counting of the bound gentamicin provides a measure of the proportion of bound drug which is radioactively labelled. This measurement leads to the calculation of the amount of gentamicin in the original sample.



Several of the methods which have been developed for gentamicin radioimmunoassay are outlined in Table 7.

Griffiths and co-workers<sup>88</sup> have compared the <sup>3</sup>H-gentamicin, <sup>125</sup>I-gentamicin, radioenzyme, and microbiological assay methods. Jonsson<sup>89</sup> studied the specificity of the <sup>125</sup>I-gentamicin radioimmunoassay and compared the relative responses of the individual gentamicin components to the responses of several aminoglycoside antibiotics. Similar comparisons have been made for the <sup>3</sup>H-gentamicin radioimmunoassay.<sup>90,91,92</sup>

#### 10.4 Radioenzyme Assay

Determination of gentamicin in serum by radioenzyme assay is based on the enzyme-catalyzed derivatization of the drug with a labelled substituent group. Derivatized gentamicin is adsorbed onto some stationary medium to separate it from unreacted components. The radioactivity of the adsorbent plus gentamicin thus provides a measure of the amount of drug present in the sample.

Smith *et al.*<sup>97,98</sup> employed an R-factor-mediated enzyme to adenylylate gentamicin with <sup>14</sup>C-labelled ATP serving as the source of the adenylyl group. Adenylylated drug, but not ATP, was adsorbed onto Whatman P-81 phosphocellulose paper. Furger *et al.*<sup>99</sup> used a similar technique where the labelled material was <sup>3</sup>H-ATP. Smith and co-workers<sup>100</sup> also reported the isolation, partial purification, and characterization of the gentamicin adenine mononucleotide transferase, as well as comparing methods in which the sources of the adenylyl group were <sup>14</sup>C-ATP and  $\alpha$ -<sup>32</sup>P-ATP. The <sup>14</sup>C-labelled enzyme assay has also been compared to the microbiological assay.<sup>100,101,102</sup>

O'Neill *et al.*<sup>103</sup> as well as Broughall and Reeves<sup>104</sup> have compared enzyme assays involving adenylylation to those employing acetylation. Two additional papers<sup>105,106</sup> have described radioenzyme assays in which the substituent group was acetyl derived from <sup>14</sup>C-acetyl coenzyme A.

#### 10.5 High Pressure Liquid Chromatography.

Anhalt *et al.*<sup>79,80</sup> described a high pressure liquid chromatography (HPLC) procedure for the assay of gentamicin in serum. The technique involves the separation of gentamicin from interfering compounds in serum on a CM-

Table 7

Radioimmunoassay Methods for Gentamicin

<u>Carrier Protein</u>	<u>Source of Antibody</u>	<u>Separation Method</u>	<u>Isotope</u>	<u>Sensitivity</u>	<u>Reference</u>
Human serum albumin	Rabbit	dextran:charcoal adsorption	$^3\text{H}$ , $^{125}\text{I}$	---	88
Human serum albumin bovine serum albumin porcine thyroglobulin keyhole limpet hemo- cyanin	Rabbit	second antibody precipitation	$^3\text{H}$	2 ng	93,94,95
Human serum albumin	Rabbit	killed protein-A containing <u>Staphylococcus aureus</u>	$^{125}\text{I}$	---	89
Bovine serum albumin	Rabbit	dextran:charcoal	$^3\text{H}$	80 pg	90,96
Bovine thyroglobulin	Rabbit	dextran:charcoal adsorption	$^3\text{H}$	10 ng	91
Human serum albumin	Rabbit	dextran:charcoal adsorption	$^3\text{H}$	0.01 $\mu\text{g/ml}$	92

Sephadex column followed by analysis using reverse phase ion-pair chromatography. 1-N-acetylgentamicin C<sub>1</sub> may be used as internal standard for the serum assay. Continuous-flow, post-column derivatization with o-phthalaldehyde is used to form fluorescent products for detection. The HPLC technique, when compared to microbiological assays, offers advantages with regard to rapidity, specificity and precision.

#### 11. Acknowledgements

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# HALOPERIDOL

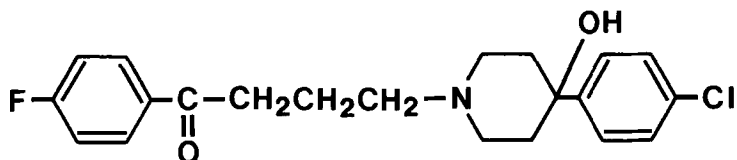
*Casimir A. Janicki and Chan Yan Ko*

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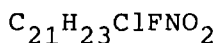
## 1. Description

### 1.1 Name, Formula, Molecular Weight

Haloperidol is 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidiny]-1'-(4-fluorophenyl)-1-butanone. The trademark of the manufactured dosage form is HALDOL®.



The molecular weight is 375.87



### 1.2 Appearance, Color, Odor

White to faintly yellowish amorphous or microcrystalline odorless powder.

## 2. Physical Properties

### 2.1 Infrared Spectrum

The infrared spectrum is presented in Figure 1. The spectrum was obtained from a potassium bromide dispersion using a Perkin-Elmer Model 283 grating infrared spectrophotometer. A list of the assignments made for some of the characteristic bands is given in Table I (1,2).

Table I  
IR Spectral Assignment for Haloperidol

3125	-OH
2953	-CH <sub>2</sub>
2918	
2839	
2822	

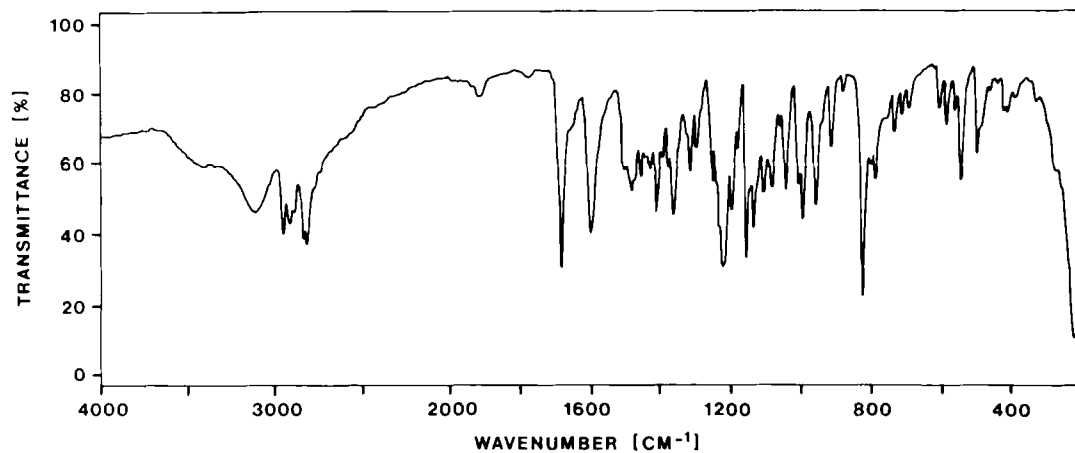


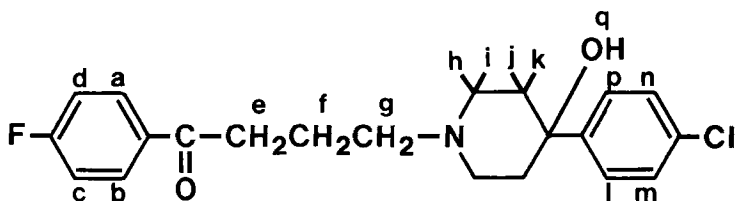
FIGURE 1: The Infrared Spectrum of Haloperidol, KBr Dispersion. Instrument: Perkin-Elmer 521

Table I (cont'd)

1681	-C=O
1597	Substituted Aromatic Ring
1500	
1454	
1482	-CH <sub>2</sub>
1410	
1221	-OH
1156	-CH deformation of F substituted aromatic ring
995	-Cl substituted aromatic ring
827	-CH deformation of p-substituted aromatic ring
541	-C=O

## 2.2 Nuclear Magnetic Resonance Spectrum

The 90 MHz spectrum of haloperidol presented in Figure 2 was obtained in deuterated dimethylsulfoxide (d<sub>6</sub>) at a concentration of 82 mg/ml with tetramethylsilane as the internal standard. Spectral assignments are listed in Table II (2).



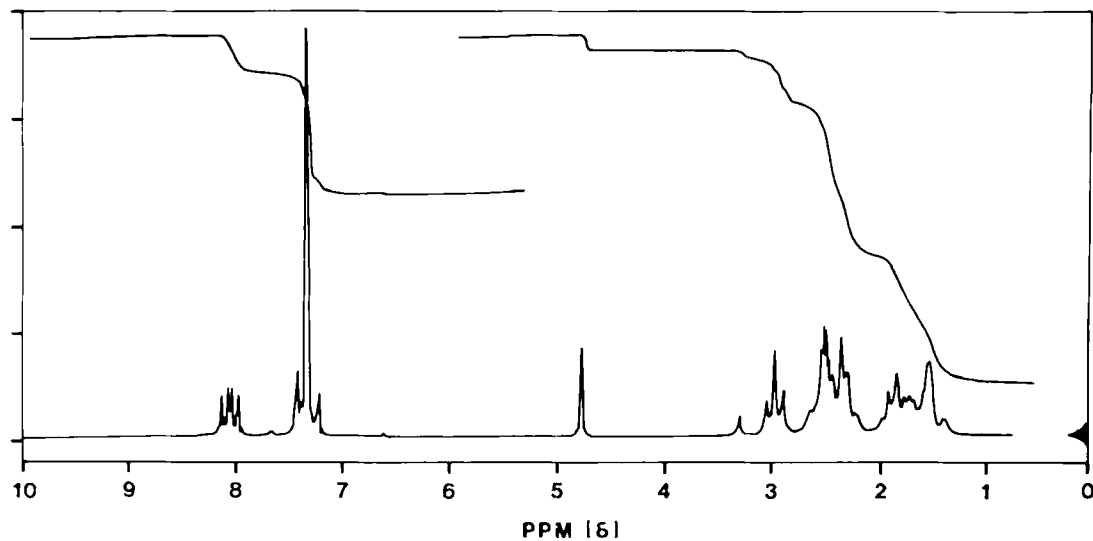


FIGURE 2: The NMR of Haloperidol in Deuterated Dimethylsulfoxide ( $d_6$ ) with TMS as the Internal Standard. Instrument: Perkin-Elmer R-32.

Table II  
NMR Spectral Assignments for Haloperidol

<u>Chemical Shift</u> <u>(ppm)</u>	<u>Multiplicity</u>	<u>Characteristic of</u> <u>proton</u>
8.05	multiplet	a,b
7.32	singlet	l,m,n,p
7.31	multiplet	c,d
4.77	singlet	q
2.98	triplet	g
2.20-2.70	multiplet	e,h,i
1.38-1.97	multiplet	f,j,k

### 2.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of haloperidol obtained from a 9:1 0.1 M hydrochloric acid: methanol solution is shown in Figure 3. Two absorption maxima, were observed at 245 nm and 221 nm, with molar absorptivities of about 13,300 and 15,000 respectively.

### 2.4 Mass Spectrum

The EI mass spectrum, is given in Figure 4, and the fragmentation pattern is presented in Table III. The fragmentation patterns were discussed in further detail in papers by Blessington (3), Leferink and Moes (4), and Diding and Co-workers (5).

The chemical ionization mass spectrum obtained on a Finnigan model 3300 CI mass spectrometer with a model 6100 data system using methane as reagent gas is presented in Figure 5 (6). A strong hydrogenated molecular ion was observed. The fragmentation pattern is presented in Table IV.



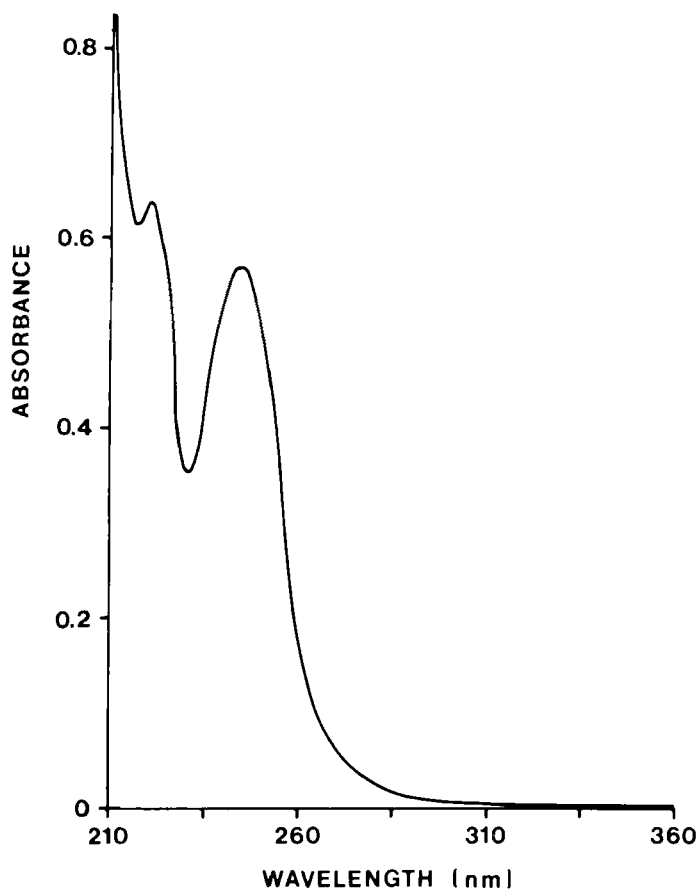


FIGURE 3: The UV Absorption Spectrum of Haloperidol in 9:1 0.1 M Hydrochloric acid: Methanol.

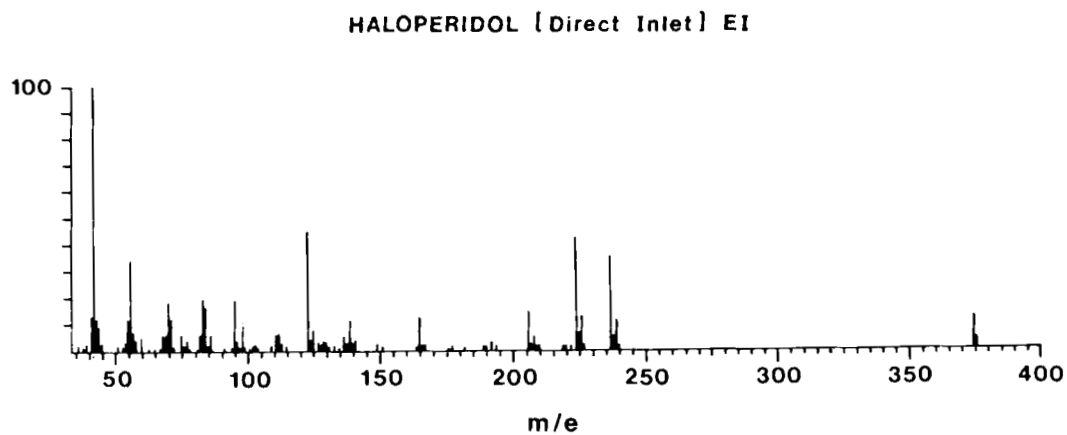


FIGURE 4: The Mass Spectrum of Haloperidol,  
Electron Impact Ionization. Instrument:  
Finnigan Model 3300 D.

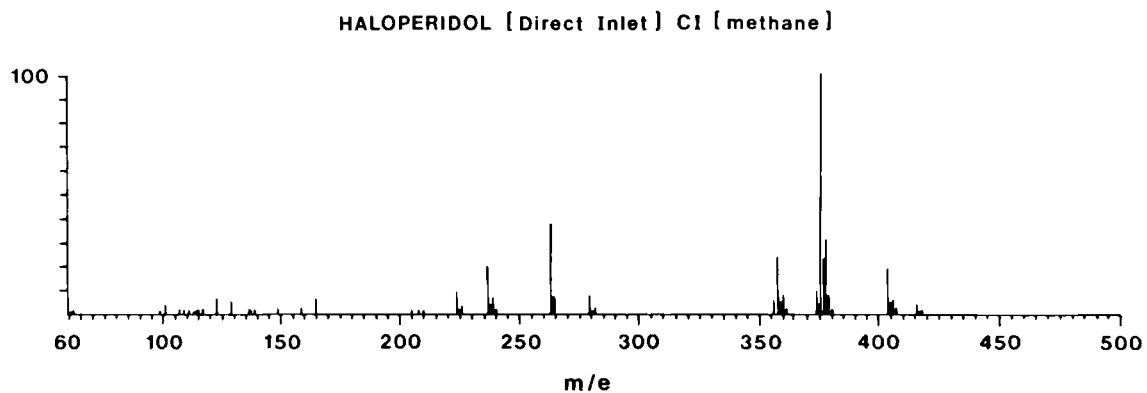
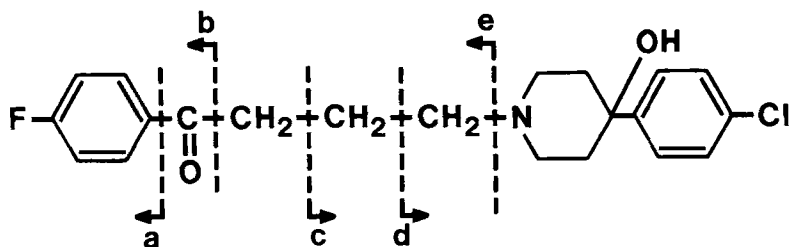


FIGURE 5: The Mass Spectrum of Haloperidol,  
Chemical Ionization with Methane  
Instrument: Finnigan Model 3300 D.

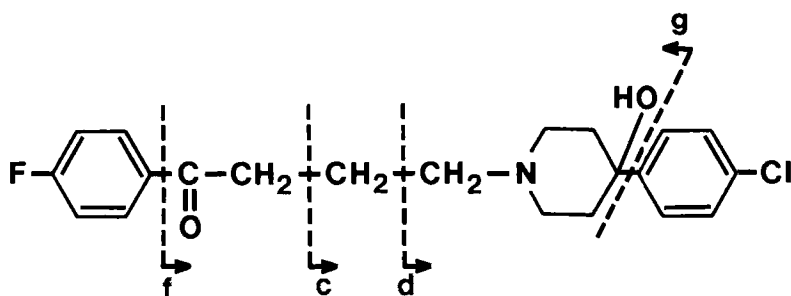
Table III  
Mass Fragmentation Pattern of Haloperidol on EI-MS



<u>M/e</u>	<u>Ion</u>
375	M+
237	C-H
224	d
206	d-H <sub>2</sub> O
165	e
123	b
95	a

Table IV

Major Ions and Fragmentation of the CI-MS of  
Haloperidol Methane as the Reactant Gas.



<u>m/e</u>	<u>Ion</u>
418	$M \cdot C_3H_7^+$
404	$M \cdot C_2H_5^+$
376	$M \cdot H^+$
358	$M \cdot H^+ - H_2O$
280	f
264	g
237	c - H
224	d

The isotopic ratio of  $Cl^{35}:Cl^{37}$ , 3:1, was observed.

## 2.5 Melting Range

The melting range of a haloperidol sample, determined after drying in vacuum at 60°C for 3 hours, is between 147°-152°C according to the USP XIX class I procedure. No polymorphs of haloperidol have been reported to date.

## 2.6 Differential Scanning Calorimetry (DSC)

The DSC of haloperidol is shown in Figure 6. A melting endotherm is observed around 423°K using a temperature program of 5°/minute (7).

## 2.7 Solubility

The approximate solubilities obtained at room temperature are listed in Table V (2, 7, 11).

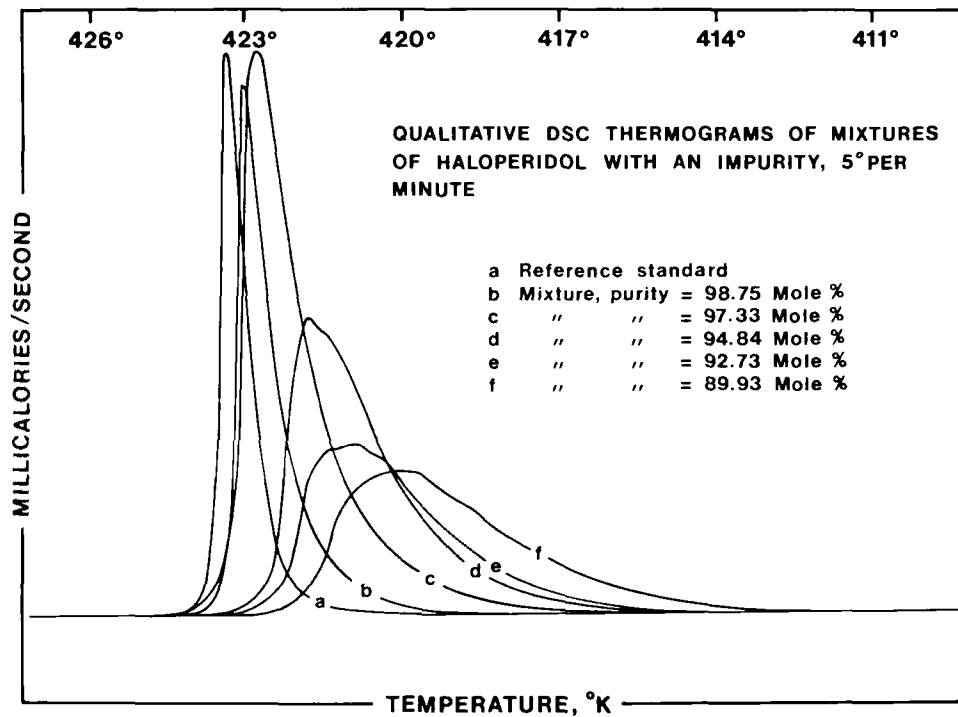


FIGURE 6: DSC Thermogram of Haloperidol. Instrument: Perkin-Elmer DSC-2.

Table V  
Solubility Data of Haloperidol at Room Temperature

<u>Solvent</u>	<u>Approximate Solubility (g/100 ml)</u>
Acetone	2.0
Benzene	1.1
Chloroform	6.6
Citric Acid (0.1 M)	1.3
Ethanol	1.7
Ether	0.5
Ethyl Acetate	1.8
n-Hexane	0.5
Lactic Acid	100
Methanol	1.8
n-Octanol	<0.1
2-Propanol	0.5
Tartaric Acid (0.1 M)	1.2
Water (pH 5.9)	<0.01

### 2.8 pKa

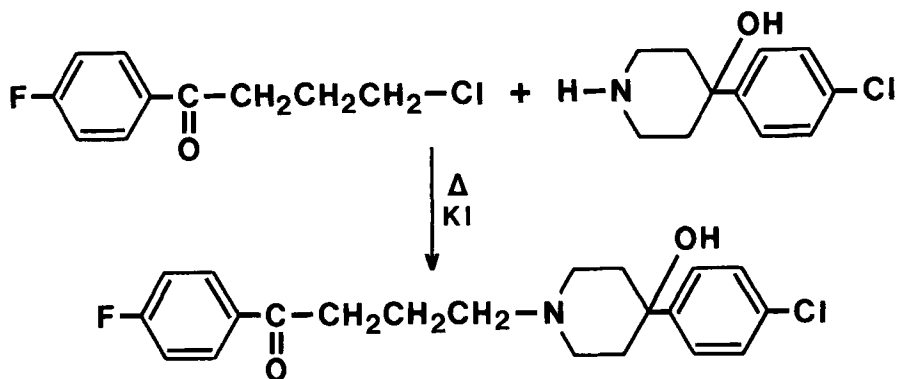
The pKa of haloperidol is 8.3 calculated by linear extrapolation using potentiometric titration in 15%, 25%, 35%, 45% methanol-water (v/v) with 0.005 N NaOH as titrant (1).

### 2.9 X-ray Diffraction

Reed and Schaefer (8) determined the crystal and molecular structure of haloperidol by single crystal X-ray diffraction techniques.

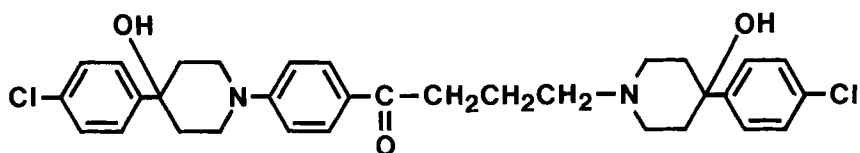
## 3. Synthesis

Haloperidol is synthesized by heating a mixture of 4-chloro-1-(4-fluorophenyl)-1-butanone, potassium iodide, and 4-(4-chlorophenyl)-4-piperidinol in a toluene solvent to about 100°C, in a closed vessel, (9,10).



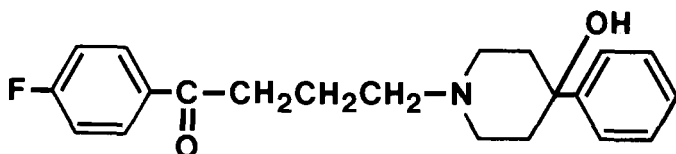
The 4-chloro-1-(4-fluorophenyl)-1-butanone was obtained by a Friedel Crafts reaction using fluorobenzene whereas the 4-(4-chlorophenyl)-4-piperidinol was obtained in three steps from  $\alpha$ -methylstyrene.

A major impurity that has been isolated and identified is 4-(4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl)-1-(4-(4-(4-chlorophenyl)-4-hydroxy-4-piperidinyl)phenyl)-1-butanone. The structure is given as:





Diding and co-workers identified deschlorohaloperidol as an impurity in a sample of haloperidol hydrochloride by GC-MS (5). The structure is given as:



#### 4. Stability Degradation

Haloperidol is a relatively stable compound. Samples have been found to be stable for up to five years stored at room temperature in amber glass containers. Storage for up to one year at 45°C in amber glass containers did not adversely affect the drug substance. Haloperidol discolors and de-grades when exposed to natural sunlight for long periods. However, no degradation was noted when the drug substance was exposed to 2000 foot candles for two weeks. The drug is only very slightly hygroscopic (2,7).

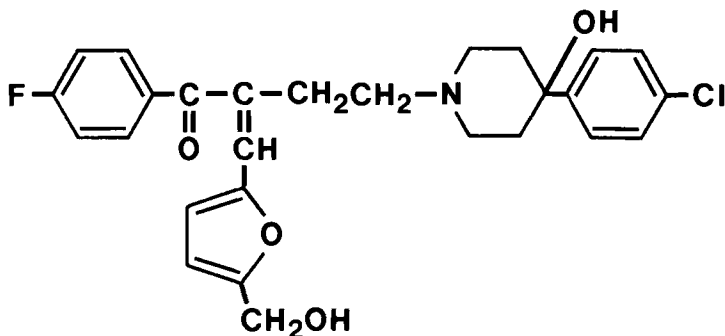
Haloperidol suspensions (1 mg/ml) were refluxed for 24 hours in water, 0.1 N sodium hydroxide, and 1 N sodium hydroxide. No degradation was observed. Under the same reflux condition but in 0.1 N hydrochloric acid solution and 1 N hydrochloric acid solution approximately 10% and 50% degradation was observed respectively (7). The hydrolysis products have not been identified.

A pH stability profile has been obtained from pH 2 to pH 8 using citrate-phosphate buffers. Good stability was observed at room temperature, 40°C and 60°C for up to 2 weeks (11).

Pharmaceutical solutions in lactic acid with a pH of about 3 have been found to be stable for up to 5 years at room temperature, 2 years at 40°C

and 6 months at 60°C. However, when the solutions were exposed to natural sunlight they became cloudy and discolored. A drop in haloperidol content was observed by assay and TLC.

Pharmaceutical tablets have been found to be stable for up to 5 years at room temperature, 2 years at 40°C and even 6 months at 60°C (7). The stability depends on the particular tablet formulation. An example was reported where haloperidol was found to be incompatible with 5-(hydroxymethyl)-2-furfuraldehyde, an impurity in anhydrous lactose (12). The adduct of haloperidol with the furfural is shown below:



## 5. Drug Metabolic Products

The excretion and metabolism of haloperidol have been studied in rats after the administration of tritium labeled haloperidol (13,14). Oxidative N-dealkylation represents the major metabolic pathway (14). The major urinary metabolite of haloperidol is N-(2-(4-fluorophenyl)acetyl)glycine (d) (13,14). Other metabolites found were 4-fluoro-γ-oxobenzenebutanoic acid (a) (13,14) and 4-fluorobenzeneacetic acid (c) (14). Only traces of unmetabolized haloperidol were found in the urine. Since the tritium label was located on the fluorophenyl ring, the metabolism of the piperidine part of the molecule was not followed.

In man, haloperidol is metabolized to 4-fluoro- $\gamma$ -oxobenzenebutanoic acid and N-(2-(4-fluorophenyl)acetyl)glycine (15). This demonstrates that the metabolism in man follows the same general pattern as that in the rat. No measurable amounts of any glutamine or glucuronic acid derivatives could be demonstrated. A trace of unmetabolized haloperidol was found in the urine.

Recently a "reduced" form of haloperidol (e) has been detected and identified in serum and urine of patients on high doses of haloperidol (16). Hydroxylation was also proposed as another pathway (17). The proposed metabolic pathway in man for haloperidol is summarized in Figure 7, with oxidative N-dealkylation representing the major metabolic path.

## 6. Methods of Analysis

### 6.1 Elemental Analysis

<u>Element</u>	<u>% Theory</u>
Carbon	67.10
Hydrogen	6.17
Chlorine	9.43
Fluorine	5.05
Nitrogen	3.73
Oxygen	8.51

### 6.2 Non-aqueous Titrimetric Analysis

The non-aqueous titration procedure is the official method listed in the United States Pharmacopeia XIX (18) for the drug substance. An accurately weighed sample of haloperidol is dissolved in glacial acetic acid. After the addition of 3 drops of p-naphtholbenzein TS, the solution is titrated with standardized 0.05 M perchloric acid to the end point.

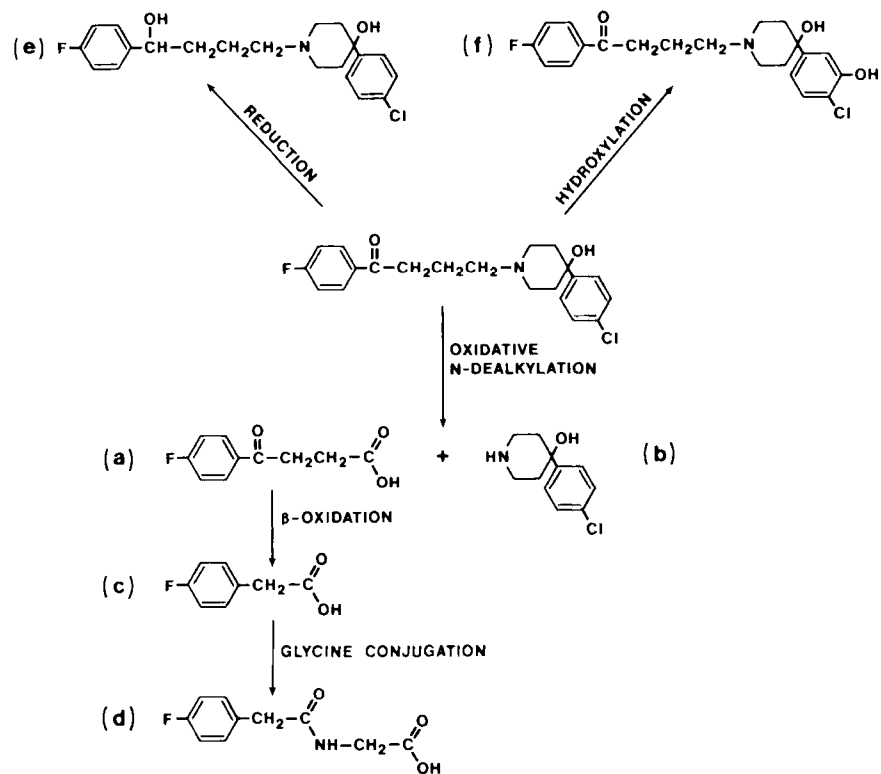


FIGURE 7: PROPOSED METABOLIC PATHWAY OF HALOPERIDOL.

### 6.3 Colorimetric Analysis

A general procedure for the quantitative determination of butyrophenones was described by Haemers and Van Den Bossche (19). Their procedure involves the reaction of butyrophenones with 3,5-dinitrobenzoic acid in an alkaline medium resulting in the formation of a red colored complex. Using this procedure, haloperidol was determined in pharmaceutical solutions and tablets. Haloperidol also forms a chloroform soluble methyl orange complex at a pH of 5, which is suitable for quantitative work (1,12). Another colorimetric procedure involves the use of potassium iodoplatinate (20). None of the colorimetric procedures have been found to be suitable for stability work since they lack the necessary specificity.

### 6.4 Spectrophotometric Analysis

The official USP XIX analysis of haloperidol tablets is a spectrophotometric analysis. A portion of powdered tablets is weighed and extracted with chloroform and 0.1 N sodium hydroxide. A portion of the chloroform layer is extracted with 0.1 N sulfuric acid and the UV spectrum recorded. The sample is compared against a reference standard diluted to the same final concentration in 0.1 N sulfuric acid, at the maximum about 245 nm.

For solutions containing parabens, the same assay described above can be used, except that the final acid layer is extracted twice with diethyl ether. This removes any UV interference in the sulfuric acid solution due to the parabens (7).

### 6.5 Thin-Layer Chromatographic Analysis

Some TLC methods for haloperidol are given in Table VI, along with the various detection methods. Braun and co-workers published a method to follow the metabolites of haloperidol in urine (13).

Table VI  
TLC Methods for Haloperidol

<u>Adsorbent</u>	<u>Solvent System</u>	<u>R<sub>f</sub></u>	<u>Ref.</u>
1. Silica GF	Ethyl Acetate: Chloroform:Methanol: Sodium Acetate Buffer (pH 4.7) (54:23:18:5)	0.64	(2)
2. Silica GF	Chloroform:Methanol (92:8)	0.40	(2)
3. Alumina GF	Chloroform:Ethanol (99:1)	0.50	(2)
4. Silica GF	Chloroform:Methanol Ammonium Hydroxide (91:8:1)	0.61	(7)
5. Silica G	Ethanol:1N hydro- chloric acid (95:5)	0.57	(7)
6. Silica G	Acetone	0.60	(21)
7. Silica G	Benzene:Acetone: Petroleum ether: Ammonium hydroxide (10:10:10:2)	0.90	(21)
8. Silica G	Acetone:Petroleum ether (7:3)	0.32	(21)
9. Silica G	n-butanol:isopropanol: Acetic Acid:Water (3:3:2:4)	0.86	(21)
10. Silica GF with Na <sub>2</sub> CO <sub>3</sub>	Methanol:Acetone (12:88)	0.50	(22)

- |   |   |      |      |
|---|---|------|------|
| 11. Silica GF<br>with Na <sub>2</sub> CO <sub>3</sub> | Ethanol:Chloro-<br>form (16:84)                                 | 0.74 | (22) |
| 12. Silica GF   | Chloroform:Methanol:<br>Formic Acid (85:10:5)                   | 0.51 | (23) |
| 13. Silica GF   | Chloroform:Methanol:<br>25% ammonium hydro-<br>xide (85:14:1)   | 0.87 | (23) |
| 14. Silica GF   | Ethylacetate:isopro-<br>panol:Ammonium hydro-<br>xide (70:25:4) | 0.81 | (24) |
| 15. Silica GF   | Cyclohexane:diethyl-<br>amine:benzene (80:15:5)                 | 0.18 | (24) |

#### 6.6 Gas Chromatographic Analysis

Haloperidol has been assayed using a glass column packed with the following: 2% OV-1 on Chromosorb W HP with a column temperature of 210°C (25); a mixture of 0.3% Versamid and 0.6% OV-17 on Gas Chrom Q with a column temperature of 230°C (26); 3% OV-17 on Gas Chrom Q with a column temperature of 280°C (27); and 3% OV-1 on Gas Chrom Q with a column temperature of 240°C (28). All of these reported methods use an electron capture detector to have the necessary sensitivity for determining haloperidol in body fluids. Bianchetti and Morselli used a 3% OV-17 on Chromosorb W at a column temperature of 285°C along with a Nitrogen-Phosphorous detector (29).

#### 6.7 High-Performance Liquid Chromatographic Analysis

A HPLC method was described in the literature (30). A reverse phase column was used with a solvent mixture of 44% tetrahydrofuran and 0.75% phosphoric acid in

water. An UV detector at 254 nm was used. A retention time of 5.5 minutes was reported, but no specificity data were given.

A HPLC method has been developed, which gives the necessary specificity, that can be used to follow the stability of haloperidol in tablets and oral and injectable solutions (31). A 10 mg equivalent of haloperidol is transferred to a 120 ml bottle. A 50 ml aliquot of chloroform and 25 ml of 0.1 N sodium hydroxide are added. The bottle is shaken for 30 minutes, centrifuged, and the aqueous layer discarded. A 15 ml aliquot of the chloroform layer is evaporated to dryness in a stream of nitrogen. The residue is dissolved in 4 ml of the internal standard solution (0.6 mg/ml oxatomide<sup>1</sup> in methanol). A 5  $\mu$ l sample is injected into the column.

The chromatographic conditions are as follows:

Column: LiChrosorb RP-18; 25 cm x 2.1 mm id  
stainless steel column  
Mobile Phase: 30% 0.1 M ammonium carbonate: 70%  
methanol  
Flow Rate: 1 ml per min  
Wavelength: 270 nm  
Retention times: Haloperidol - 3.9 min  
Oxatomide - 10.0 min

The specificity of the method is demonstrated by the data given in Table VII.

---

<sup>1</sup> 1- 3-[4-(diphenylmethyl)-1-piperazinyl]propyl -  
1,3-dihydro-2H-benzinidazol-2-one.



Table VII

Separation of impurities, degradation products and preservatives from haloperidol by HPLC.

<u>Compound</u>	<u>Retention Time</u> <u>(minutes)</u>
1) 4-Fluorobenzoic acid	1.0
2) 4-fluoro- $\gamma$ -oxobenzenebutanoic acid	1.1
3) Methylparaben	1.3
4) Propylparaben	1.9
5) 4-(4-Chlorophenyl)-4-piperidinol	2.0
6) 1-(4-Fluorophenyl)-4-(4-hydroxy-4-phenyl-1-piperidinyl)-1-butanone	2.5
7) 4-chloro-1-(4-fluorophenyl)-1-butanone	2.9
8) Haloperidol	3.9
9) Oxatomide	10.0
10) 4-(4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl)-1-(4-(4-chlorophenyl)-4-hydroxy-4-piperidinyl). phenyl)-1-butanone	11.3
11) 4-[4-(4-Chlorophenyl)-3,6-di-hydro-1(2H)-pyridyl]-4'-fluorobutyrophenone	22.0

#### 6.8 Differential Scanning Calorimetric Analysis

A quantitative analysis of the purity of haloperidol can be obtained by DSC (7), using the method by Plato and Glasgow (32). Qualitative DSC thermograms of mixtures of haloperidol with an impurity are given in Figure 6 to show the specificity of the technique.

#### 6.9 Polarographic Analysis

A polarographic analysis of haloperidol was described by Volke et al using a dropping mercury electrode (33). A half-wave potential of -1.28 volts was obtained in a

solvent of 1:1 v/v acetate buffer, pH 5.3; DMF, and -1.64 volts using 1:1 v/v 0.25 M potassium hydroxide; ethanol as the solvent.

#### 6.10 Fluorescence Analysis

Haloperidol exhibits weak fluorescence in methanol, ethanol, and 2-propanol with an excitation wavelength at 310 nm and emission wavelengths at 410 nm, 375 nm and 390 nm respectively (34). Haloperidol is converted into a strongly fluorescent derivative by the action of potassium permanganate on its alcoholic solution in an acid medium. An excitation wavelength of 305 nm produces emission at 383 nm (35).

### 7. Determination in Biological Fluids

Several gas chromatographic assay methods have been published for the determination of haloperidol in biological fluids (25,26,27,28,29) and they were given in Section 6.6.

Problems have been reported with the various gas chromatographic assays, for example, as the column temperature increased, the haloperidol dehydrated. As the temperature reached about 285°C, the haloperidol peak decreased in size while the dehydrated product peak greatly increased. At 285°, the large dehydrated-haloperidol peak was lost in the solvent peak (36). It is important that the temperature of the column stay about 240°C.

The GC assay methods often lack the required sensitivity or specificity to obtain reproducible plasma data from clinical studies. So a sensitive and specific GC/CI-MS method was developed for the determination of haloperidol in plasma (37). The procedure involves the addition of the internal standard (the chloro substituted analog of haloperidol), alkalization of the sample with sodium hydroxide and extraction with heptane containing 1.5% isoamyl alcohol. Prior to injection, the

organic layer is evaporated to dryness and reconstituted in methanol containing 5% triethylamine. The GC conditions are as follows:

Instrument: A gas chromatograph equipped with all glass-lined transfer lines

Column: 10% OV-1 on Chromosorb WHP-0.3 m x 2 mm id Column Temperature: 260°

Carrier: Methane at 25 ml/min

Detector: Quadrapole mass spectrometer set to monitor ion  $m/e$  376 ( $MH^+$  of haloperidol and  $MH^+ - H_2O$  of the internal standard)

Haloperidol has a retention time of 0.7 minutes and the internal standard of 1.1 minutes. The response curve was linear between 0.2 ng and 6.0 ng.

Radioimmunoassays have been published (17, 38,39). A radioimmunoassay kit is also available<sup>2</sup> (17) which is specific for haloperidol in the presence of the metabolites a,b,c,d, and e given in Figure 7. The limit of detection of the assay is reported to be 0.02 ng contained in 0.5 ml of plasma.

## 8. Determination in Pharmaceuticals

Demoen (1) proposed a spectrophotometric assay. However, no specificity data were given. A method that has been found to be suitable for stability work is that given in the U.S.P. XIX for tablets (18). The method has been described in Section 6.4. Janicki and Almond (12) used that method to determine the haloperidol content in direct compression tablets that contained an unexpected degradation product of haloperidol.

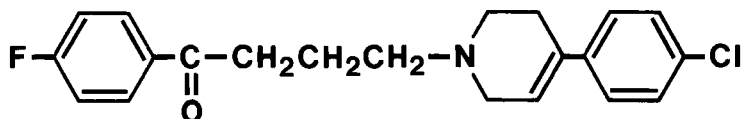
The method can also separate and quantitate haloperidol in the presence of the following compounds considered model degradation products:

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<sup>2</sup> Institut National Des Radioelements,  
6220 Fleurus, Belgium

4-chloro-1-(4-fluorophenyl)-1-butanone; 4-fluoro-oxobenzene butanoic acid; 4-fluorobenzoic acid; and 4-(4-chlorophenyl)-4-piperidinol.

The method cannot separate the dehydrated product of haloperidol from haloperidol. The structure of the dehydrated product is given as:



The dehydrated product of haloperidol is a theoretical degradation product and has not been found to be an actual degradation product in dosage forms to date (7). However, TLC solvent systems 2 and 4 in Section 6.5 can separate the dehydrated product from haloperidol, which runs ahead of haloperidol in both systems.

The HPLC assay may be used for stability analysis. The method and specificity were described in Section 6.7.

The methyl orange procedure described by Janicki and Almond (12), the colorimetric procedure using 3,5-dinitrobenzoic acid described by Haemers and Van Den Bossche (19), and the colorimetric determination using potassium iodoplatinate described by Pawelezyk and Plostkowiak (20) cannot be used for stability work since they all lack the necessary documentation of specificity. The fluorescence method of Baeyens and De Moerloose (35) has been applied to dosage forms but no specificity data were presented.

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# KHELLIN

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## 1. Description

### 1.1 Nomenclature

#### 1.1 1 Chemical Names

- a. 4,9-Dimethoxy-7-methyl-5 H-furo[3,2-g]  
-[1] benzopyran-5-one.
- b. 5,8-Dimethoxy-2-methyl-4,5-furo-6,7-  
chromone.
- c. 5,8-Dimethoxy-2-methyl-6,7-furano-  
chromone.
- d. 4,9-Dimethoxy-7-methyl-5-oxofuro  
[3,2-g] 1,2-chremone
- e. 4,9-Dimethoxy-7-methyl-5-oxofuro  
[3,2-g] [1] benzopyran.
- f. 4,9-Dimethoxy-7-methyl-5-oxo-1,8-  
dioxabenz [f] indene.

The CAS Registry No. is [82-02-0].

#### 1.1 2 Generic Name

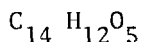
Khellin

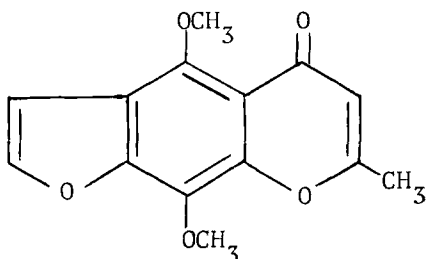
#### 1.1 3 Trade Names

Ammincardine, Amicardine, Ammipuran, Ammivin  
Ammivisnagin, Benecardin, Corafurone, Cardio-  
khellin, Ceronin, Eskel, Kelamine, Kelicorin,  
Kelicor, Keloid, Khellin, Gynokhellin, Khel-  
fren, Lynamine, Methafrone, Norkel, Simes  
Kellina, Visaminin, Visnagin, Visnagalin,  
Vasokellina, Viscardin.

## 1.2 Formulae

### 1.2 1 Empirical



1.2 2 Structural

The structure of Khellin has been elucidated by degradative methods as well as by its partial synthesis by reconstruction of the chromone ring starting from Khellinone (1) and also by its total synthesis.

1.2 3 Wiswesser Line Notation

· T C 566 DO JV MOJ BO1 HO 1L

1.3 Molecular Weight

260.24

1.4 Elemental Composition

C, 64.61%; H, 4.65%; O, 30.74%.

1.5 Appearance, Color, Taste, Odour

White odourless crystals sometimes with slight yellowish tinge, and with a bitter taste.

2. Physical Properties2.1 Crystal Properties:2.1 1 X-Ray Diffraction:

Khellin forms monoclinic-Prismatic crystals with forms {001}{100}{201}{010}{101}. From measurements  $a:b:c = 2.462:1:2.681$ ;  $B=102^{\circ}53'$ . Cleavage parallel (100) good, (010) less perfect. Optical constants:  $\alpha = 1.478$ ;  $B=1.741$ ;  $\gamma = 1.785$ . Orientation  $\alpha=b$ ; Optical axes observed on (010), dispersion  $\delta)8.2J$  (for

yellow light)  $48^{\circ} 52'$ , angle  $c: \gamma = 6^{\circ}(\text{red}), 7^{\circ}(\text{yellow}), 8\frac{1}{2}^{\circ}(\text{green}), 10\frac{1}{2}^{\circ}(\text{blue}), 12^{\circ}(\text{violet})$ .  
 Unit, cell for orientation  $a:b:c = 2,007:1:1.608$ ;  $B = 93^{\circ} 39'$ ;  $a = 14.49$ ,  $b = 7.22$ ,  $c = 11.61$  Å;  
 $Z = 4$ , space group  $C_{2h}^2 - PZ_1/n$  (derived from Polany rotation and Weissenberg diagram) (2).

### 2.1 2 Melting Range

Khellin melts at  $154-155^{\circ}$ . Boils at  $180-200^{\circ}$  at 0.05 mm Hg.

### 2.2 Solubility

25 mg/100 ml of water, 2.6 g/100 ml of methanol, 1.25 g/100 ml of isopropanol, 0.5 g/100 ml of ether, 0.15 g/100 ml of skellysolve B, and much more soluble in hot water and hot ethanol (3).

### 2.3 Identification

1. Khellin gives a red-violet color with NaOH or KOH or m-dinitrobenzene in alkaline solution(4-6).
2. A solution containing khellin 0.5-1 ml is heated to boiling with 0.3 ml of 2,4-dinitrophenylhydrazine (0.5% solution in 1.5 N hydrochloric acid) for 30 minutes, after cooling, 0.5 ml 30% potassium hydroxide and 1-2 ml of ethanol are added. The resulting red-violet stable color is proportional to the amount of khellin present. The 2,4-dinitrophenyl hydrazone of khellin was prepared and its m.p. is,  $284-285^{\circ}$ (7).
3. Add 2 ml of phosphoric acid to 0.1 g of khellin, orange-red crystals are formed, which dissolve on heating. To the viscous orange solution of 0.5 g of khellin in 1 ml phosphoric acid, gradually add dry ethylacetate with trituration to obtain yellow crystalline precipitate of the oxonium phosphate; m.p.  $126^{\circ}$  - with decomposition (8).

### 2.4 Spot Tests

1. To a few crystals of khellin on a white porcelain plate add 2 drops of phosphoric acid.(sp.gr. 1.75), an orange-colored crystals are formed (8).

2. To a few crystals of khellin add few crystals of alloxan, 5 drops of sulphuric acid and triturate with a thin glass rod till the solids dissolve, an initial orange color is formed which changes to violet color with an orange edge after 20 minutes (8).
3. This is a more specific test for khellin and is not given by compounds devoid of 5-OH and 8-OCH<sub>3</sub> substituents. The reaction is carried out by dissolving a few crystals of khellin in HNO<sub>3</sub>, and then destroying the oxonium salt by diluting with alkali to yield a color, which is due to the quinone formed by oxidation with HNO<sub>3</sub> (9,10).

## 2.5 Microcrystal Tests

Khellin gives oxonium salt compounds when the following reagents are added to the dry substance (11):

1. I<sub>2</sub>-KI reagent, gives trimorphic crystallisation. In the outer zone of the precipitation there are rather coarse needles of good birefringence, those broad enough showing a yellow to dark red dichroism; then a zone of small grains, some dichroic, yellow to dark, mostly nondichroic, red after the test has stood a little while, at the central concentrations fine needles in purple masses, some of these needles in outer tufts showing dark blue to light brown dichroism. This is a sensitive test and probably specific.
2. HClO<sub>4</sub>, gives yellow rods and needles.
3. HAuBr<sub>4</sub> in dil HClO<sub>4</sub>-HOAc gives fine needles, and at central concentration large tablets, chips and bars and fans of fairly large needles, with yellow to orange dichroism.

## 2.6 Spectral Properties

### 2.6.1 Infrared Spectrum

The infrared spectrum of khellin is recorded as a nujol mull on a Unicam SP 1025 spectrophotometer and is shown in Fig. 1. The assignments

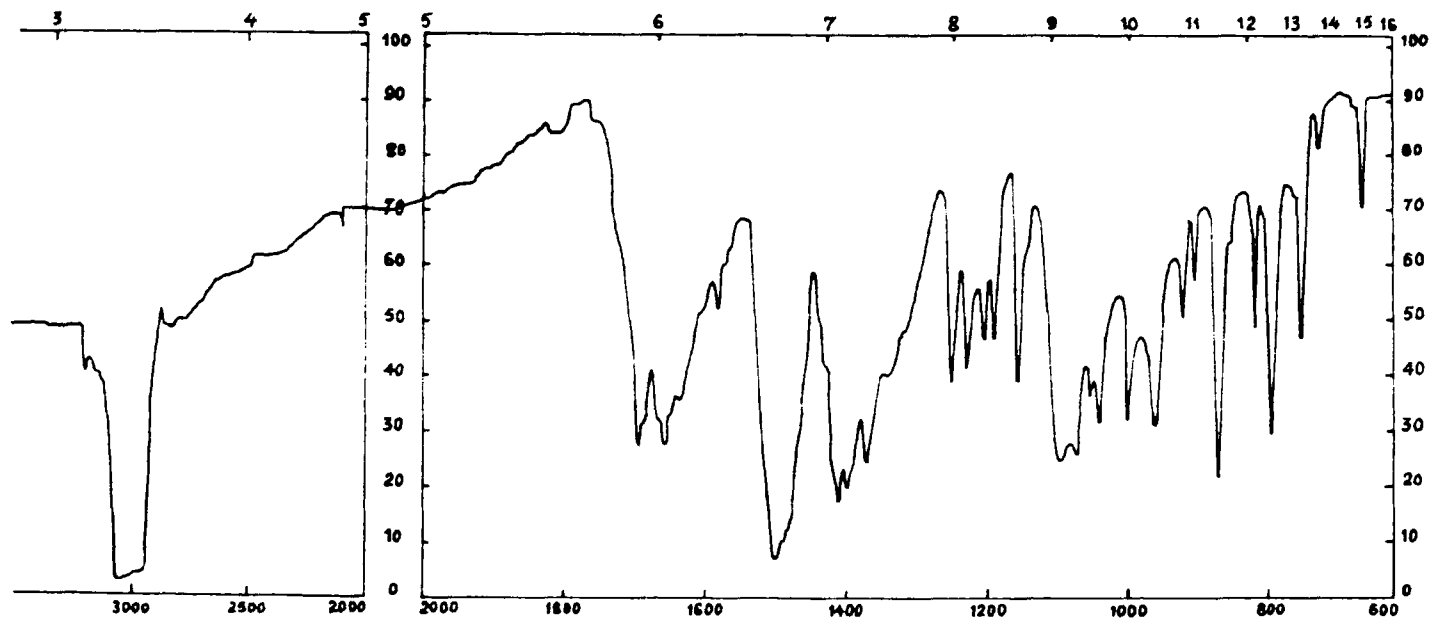


Fig. 1: Infrared spectrum of khellin in nujol

for the characteristic bands in the infrared spectrum are listed in table 1.

Table 1

<u>Frequency <math>\text{cm}^{-1}</math></u>	<u>Assignment</u>
1690	C = O
1650,1640	C = O
1600	C = C (aromatic)
1580	ethylenic linkage
1250,1230	
1190,1160	C-O-C
1090,1070	
870,820	-CH out of plane
790,740	deformation.
720.	

Other fingre print bands characteristic of khellin are 1390,1370, 1050, 1040, 1000, 960, 920 and 910.

## 2.6 2 Ultraviolet Spectrum (UV)

The UV spectrum of khellin in ethanol was scanned using Pye Unicam SP 800; from 400-200 nm, three maxima and three minima were observed. The maxima are located at 220, 244 and 328 nm. The minima occur at 232, 272 and 300 nm. The spectrum is shown in Fig.2. The UV spectral data of khellin and analogues have also been reported (12).

## 2.6 3 Nuclear Magnetic Resonance Spectrum (NMR)

### Proton Spectrum

The proton NMR spectrum of khellin in deuterated chloroform is shown in Fig.3. It was recorded on a Varian T-60A, 60MHz NMR Spectrometer, using tetramethylsilane as an internal reference. It is to be noted that both natural and synthetic khellin are currently used in pharmaceutical formulations. Natural khellin might contain variable amounts of visnagin due to incomplete purification.

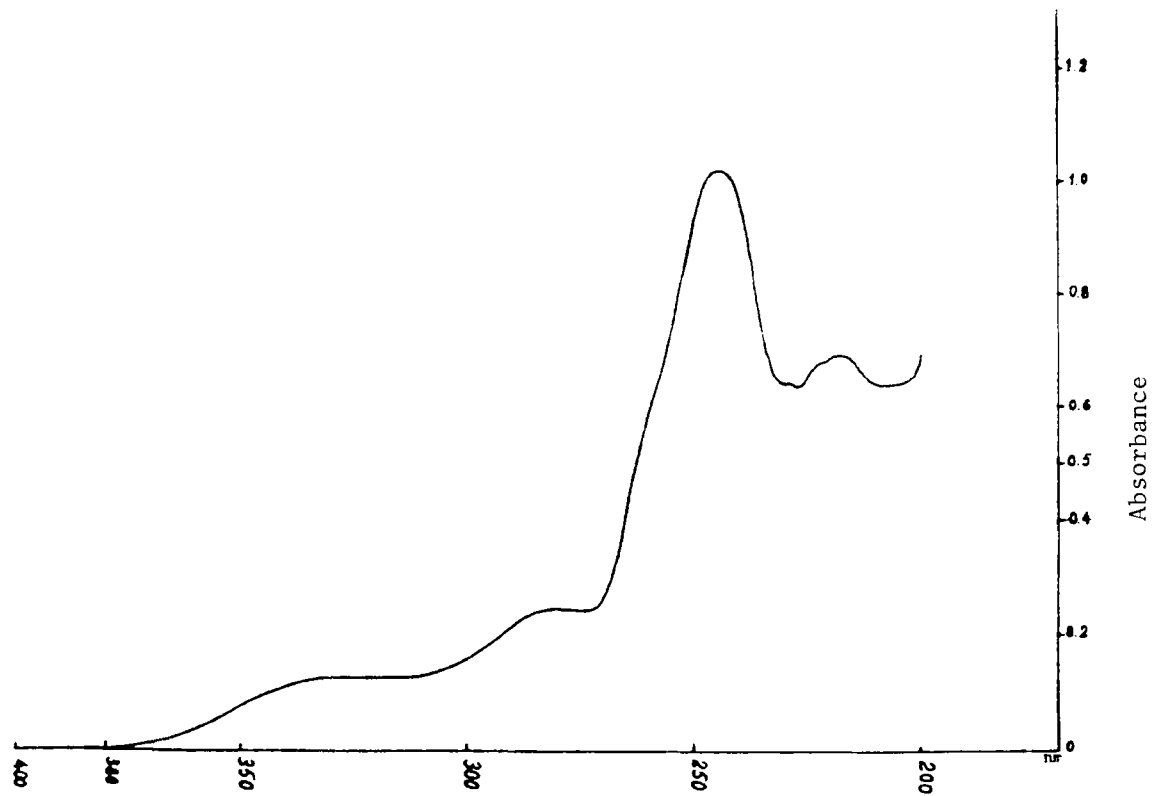


Fig. 2: Ultraviolet spectrum of khellin in ethanol.

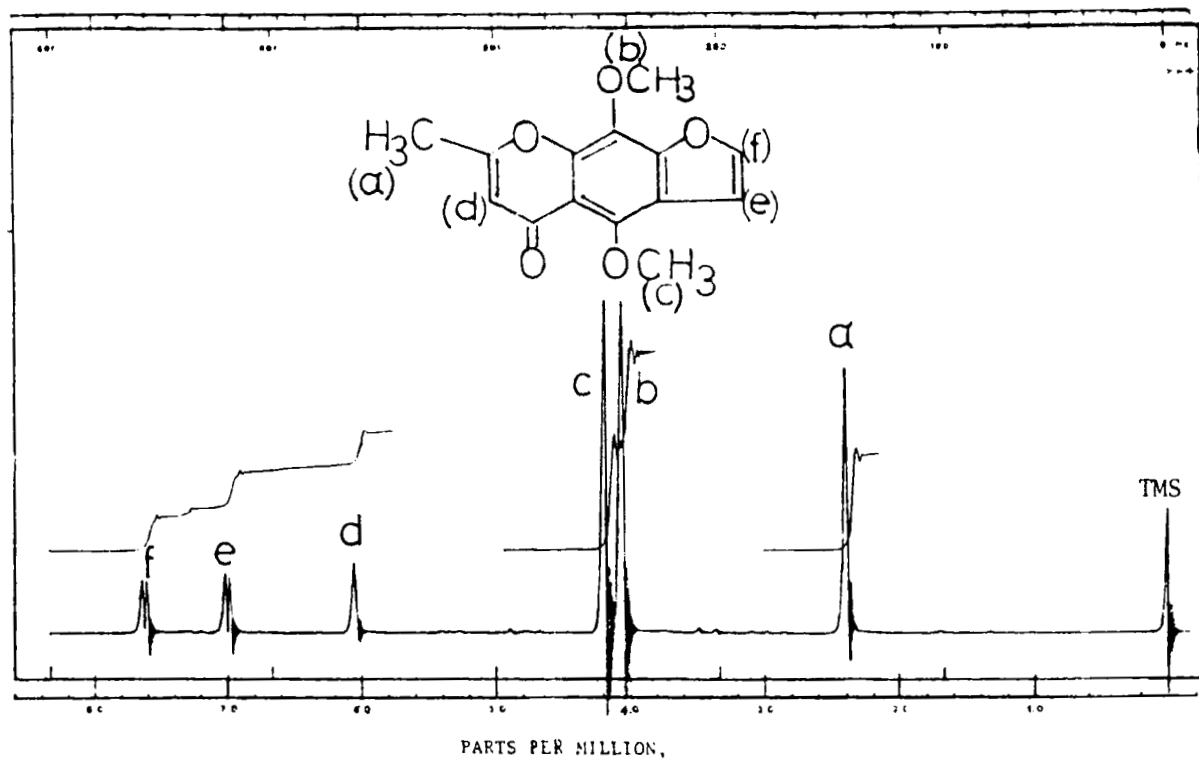


Fig. 3: PMR spectrum of khellin and TMS in  $\text{CDCl}_3$ .



Therefore, the PMR spectrum of visnagin in deuterated chloroform using tetramethylsilane is shown in Fig. 4.

The PMR spectral assignments of khellin and visnagin are given in Table 2 (13).

Table 2: PMR Comparison of Khellin and Visnagin

	Chemical shifts( $\delta$ )						
	2-CH (s)	3-H (s)	5-OCH <sub>3</sub> (s)	8-OCH <sub>3</sub> (s)	8-H (s)	2-H (d)	3-H (d)
Khellin	2.40	6.05	4.20	4.05	-	7.63	7.00
Visnagin	2.33	6.03	4.20	-	7.18	7.63	7.00

(s) = singlet, (d) = doublet.

#### 2.6 4 Mass Spectrum

The mass spectrum of khellin obtained by conventional electron impact ionization shows a molecular ion  $M^+$  at  $m/e$  260.07 (14). The  $M^+$  ion peak is the base peak and is shown in Fig. 5. The fragmentation of khellin and other furano-chromones have been reported (15).

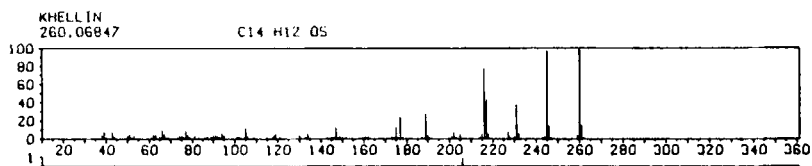


Fig. 5: Mass Spectrum of Khellin

### 3. Isolation

Khellin [5,8-Dimethoxy-2-methyl-4,5-furo-6,7-chromone] is obtained as the main chromone constituent from the fruits of *Ammi visnaga* (Fam. Umbelliferae) (16-19).

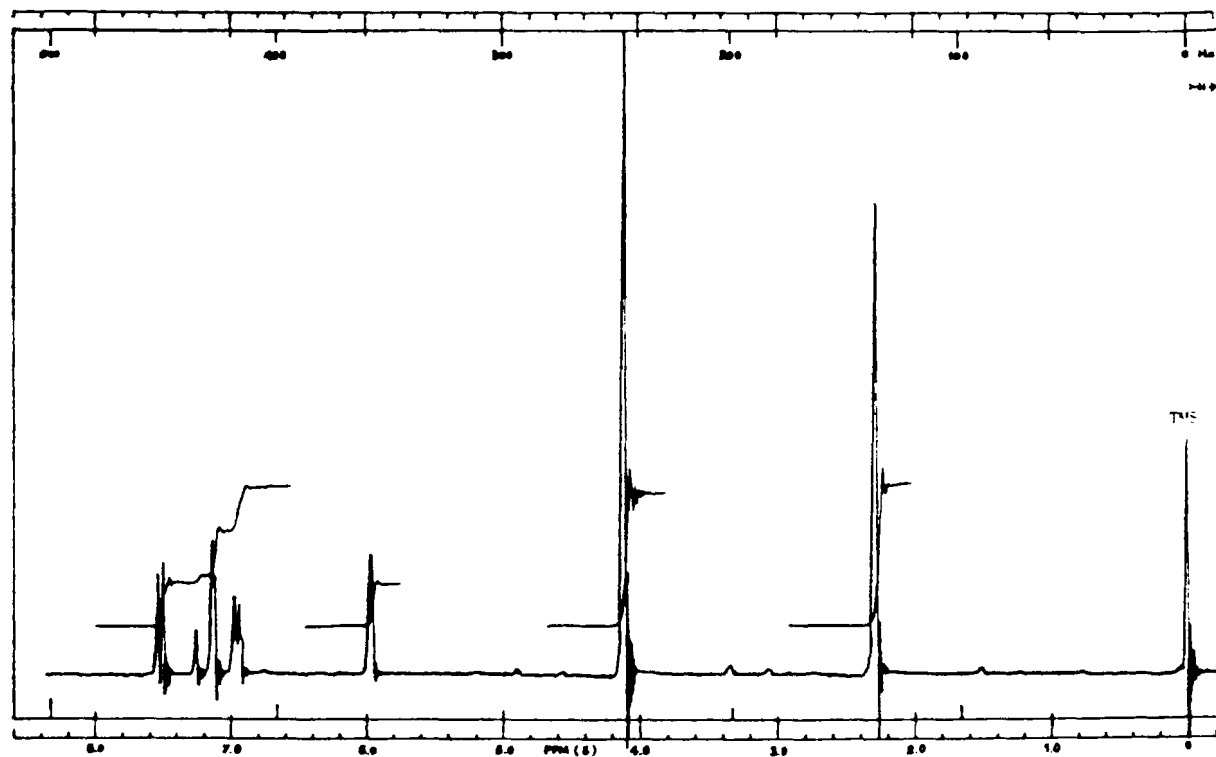


Fig. 4: PMR Spectrum of visnagin and TMS in  $\text{CDCl}_3$

#### 4. Biosynthesis

Geissman (20) on the basis of the striking similarity between the furocoumarin isopimpinellin and the furanochromone khellin, suggests that the two heterocyclic ring systems have a common origin, namely cinnamic acid and they are formed by the shikimic acid - phenylalanine pathway. Extension of the cinnamic acid side-chain, possibly at the orthohydroxylated intermediate as glucoside, by the addition of two carbon fragment, as shown.

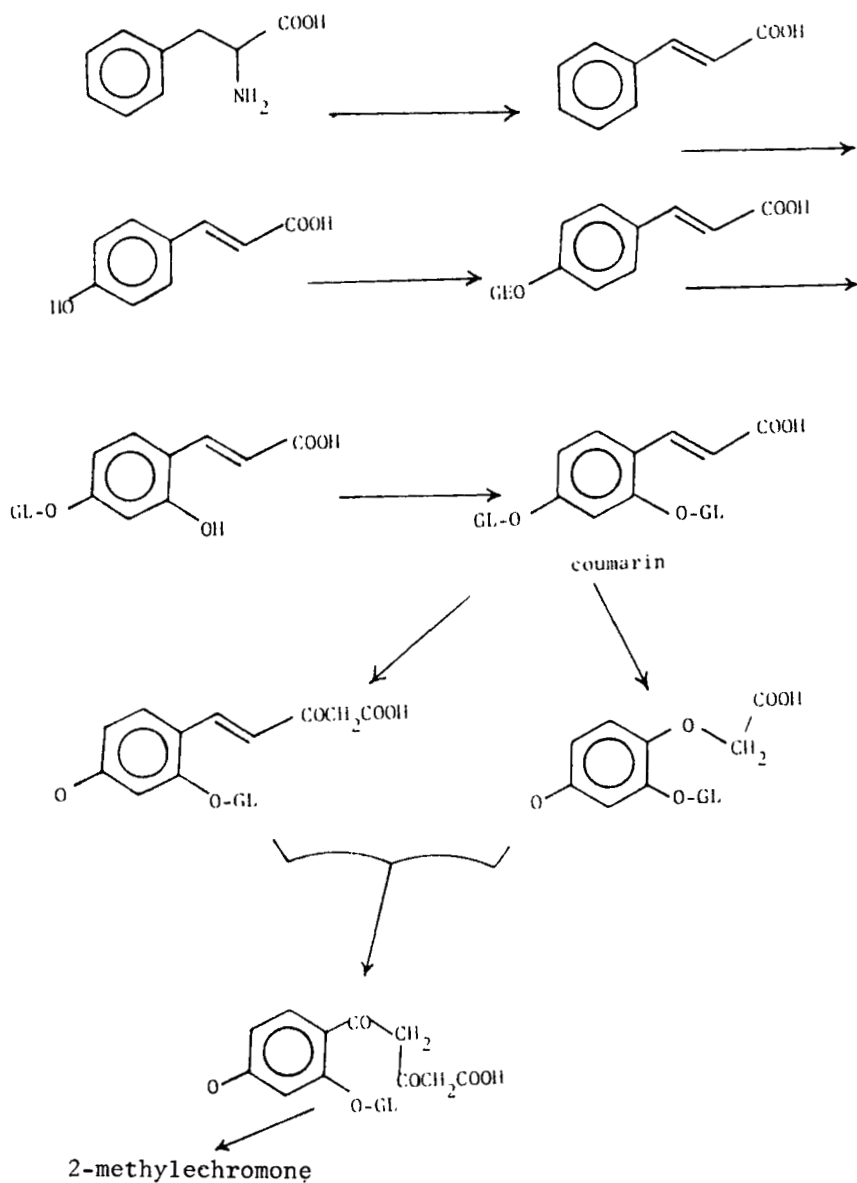
However, Chen et al (21) has reported that biosynthesis of radio-active khellin and visnagin from  $C^{14}$ -acetate by *Ammi visnaga* plants. Their results support the hypothesis that furanochromones are biosynthesised via an acetate condensation pathway rather than by the phenylalanine-shikimic acid route as is the case for the very closely related furocoumarins.

#### 5. Synthesis

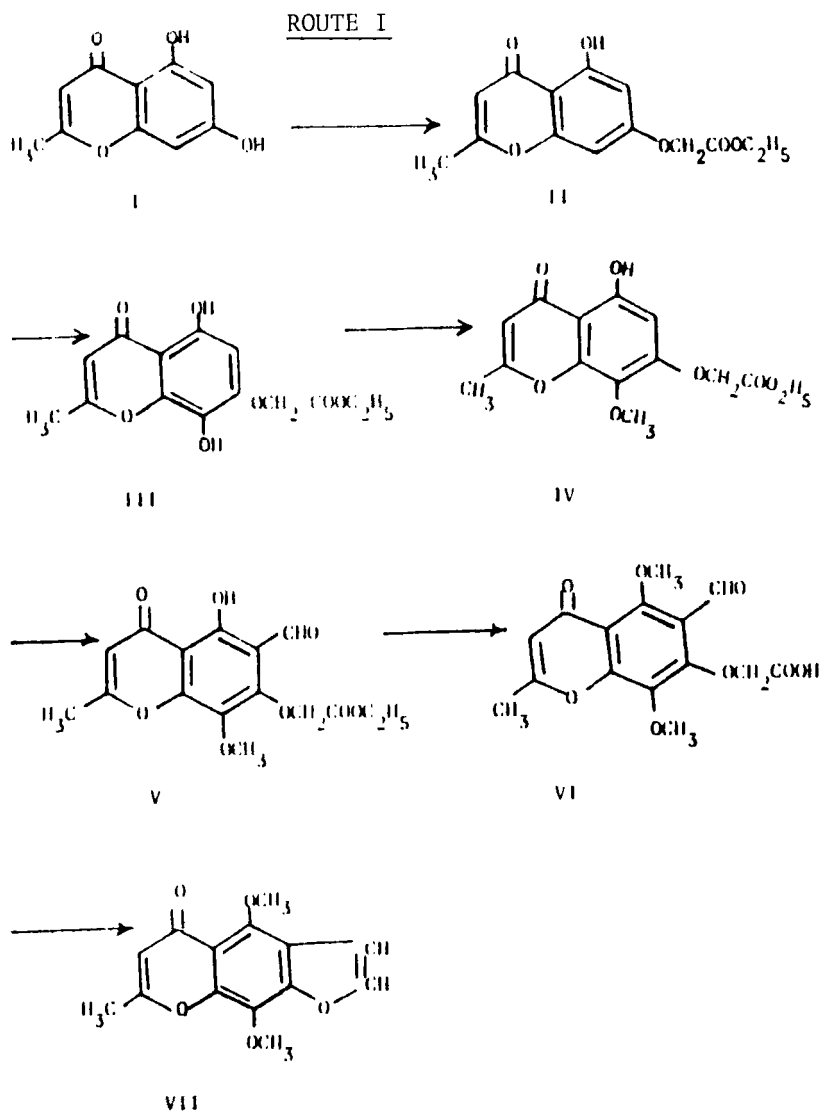
Several synthetic routes to khellin and derivatives have been reported (22-35). Two of them are illustrated.

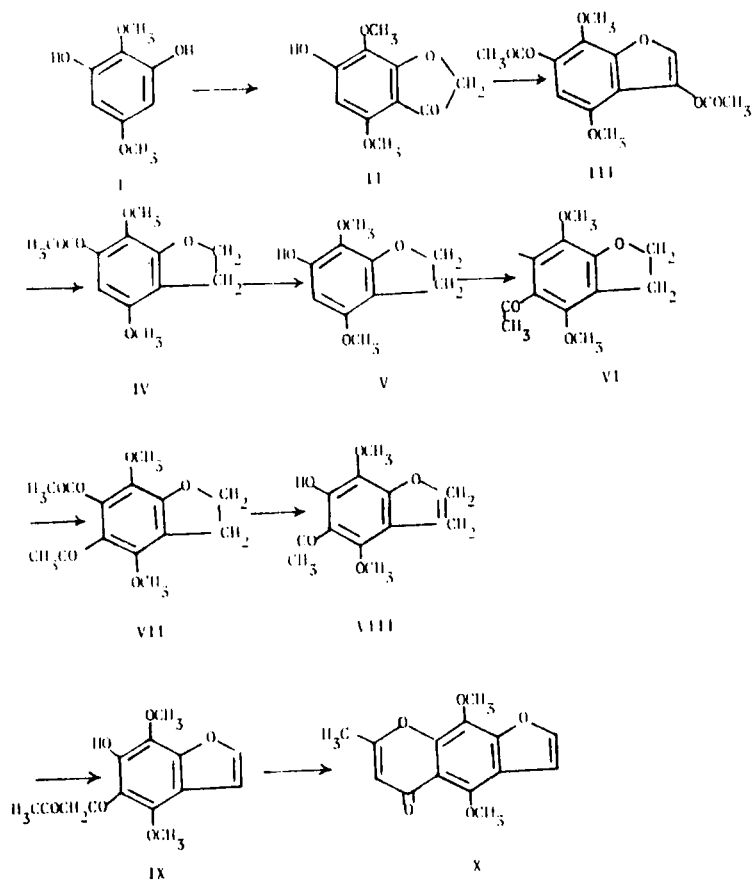
Route - I : Involves the condensation of 5,7-Dihydroxy-2 methylchromone (I) with bromoethyl acetate, followed by nuclear oxidation of the product with alkaline persulfate to give quinol (III) (36). The partially methylated quinol (IV) is condensed with hexamine to give the aldehyde (V) which on complete methylation and hydrolysis with dilute alkali gives aldehydic acid (VI), which on boiling with sod. acetate and acetic anhydride afforded khellin (VIII).

Route - II: Describes total synthesis of khellin starting with 2,5-dimethoxyresorcinol (I), (37,38). (I) is converted into the coumaranone (II) by means of Hoesch Reaction, using chloroacetanilide. Acetylation of (II) and reduction yielded (IV). After removal of the acetyl group of (IV) it was subjected to a Hoesch Reaction with acetonitrile to yield dihydrokhellinone (VI). Acetylation of VI and reaction with N-Bromosuccinimide in carbon tetrachloride and purification yielded pure khellinone (VIII). Khellinone was condensed with ethyl-acetate in presence of sodium hydride to give a diketone (IX) which is then cyclised to give khellin (X).



Biosynthesis of a 2-methylchromone.



ROUTE II

## 6. Methods of Analysis

### 6.1 Modified Zeisel-Viebock Method (39)

Malysz et al (40) have applied this method for determining methoxy groups by using a special apparatus. The sample containing equivalent of 2-5 mg of methoxy group was mixed with 0.5 g of phenol, 2.4 g of potassium iodide and 4 ml of phosphoric acid, then heated in an atmosphere of  $\text{CO}_2$  at  $150^\circ$  for 1.5 hours. The iodomethane produced was distilled off in a stream of  $\text{CO}_2$  and absorbed in 10 ml of bromine solution (dissolve 10 g of potassium acetate in 100 ml of anhydrous acetic acid and add 4 ml of  $\text{Br}_2$ ). The absorbent solution was then mixed with 10 ml of 2.5% aqueous sodium acetate and diluted with 100 ml of water and the excess of bromine was destroyed with 3 drops of formic acid. The colorless solution was acidified with 10 ml of 2N sulfuric acid, 1 g of potassium iodide was added and after 5 minutes the liberated iodine was titrated with 0.05N sodium thiosulfate solution and starch as indicator. This method has been used to determine khellin in the pure form and in pharmaceutical preparations. The results were within  $\pm 0.1$ - $\pm 1\%$  of those obtained by various pharmacopoeial methods.

### 6.2 Colorimetry

Different colorimetric methods have been used for the determination of khellin, based on color reaction with sulphuric acid (41,42), phosphoric acid (43,45) and m-dinitrobenzene and potassium hydroxide (45,47). A colorimetric method based on the color developed by treating khellin with nitric acid followed by sodium hydroxide is officially adopted by E.P. 1972 (48). The method is based on the oxidation of khellin with nitric acid to produce quinone derivative which gives violet color with sodium hydroxide solution. The reaction was favourably carried out at room temperature ( $20$ - $30^\circ$ ), lower or higher temperature, either slow the reaction or enhance it, respectively. The absorbance of the resulting violet color is measured at  $\lambda_{\text{max}}$  540 nm within 15 minutes





Table 4

<u>Solvent System</u>	<u>R<sub>f</sub> Value</u>	<u>Reference</u>
Xylene-Acetone(4:1)	0.25	51
Xylene-Ethyl Acetate-Acetic Acid (15:5:1)	0.31	51
Xylene-Ethyl Acetate-Pyridine (6:18:1)	0.62	51
Xylene-Pyridine-Formic Acid (23:5:2).	0.63	51
Ether-Formamide-ethanol (93:2:5).	0.86	52
Chloroform-Tetrahydrofuran-formamide (50:50:6.5).	0.67	52
Ethylacetate-Benzene-Water (50:50:50).	0.50	52
Ethylacetate-Benzene-water (50:75:50) (Fig.6).	0.31	52

#### 6.5 Two Dimensional Thin Layer Chromatography

Karawya et al (53), reported two dimensional TLC technique on silica gel G plates using ethylacetate as developing solvent (Fig.7). This technique has offered a better separation of khellin from visnagin than the unidimensional multiple-run technique and was used in the quantitative recovery of the two constituents and their subsequent colorimetric estimation. It is also used for the determination of khellin in pharmaceutical formulations.

#### 6.6 PMR Spectrometry

Hassan and Aboutabl (13) has published a rapid, accurate and specific PMR method for the determination of khellin in bulk drug and pharmaceutical

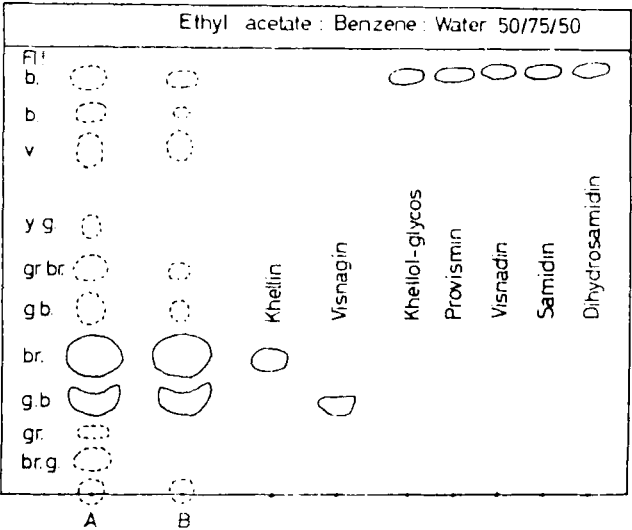


Fig. 6: A:0.1 cc alcoholic extract of Ammi visnaga fruits.

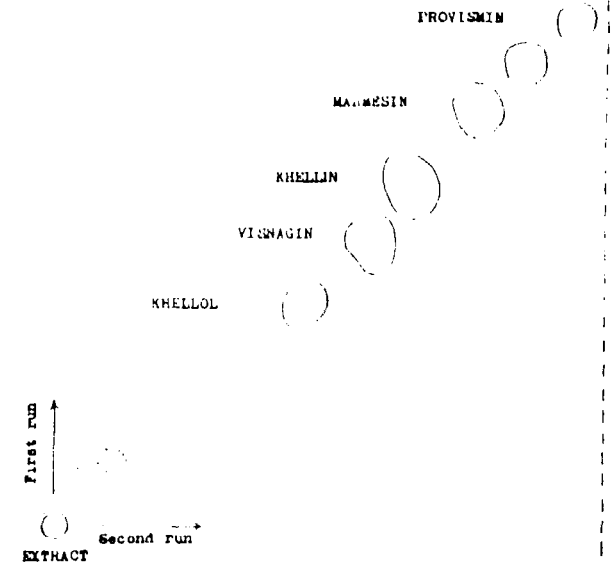


Fig. 7: Two dimensional TLC using ethyl acetate as the developing system.

formulations. It also furnishes a specific means of identification of khellin as well as simultaneous detection and determination of the less potent 8-demethoxy analogue, visnagin. Acetanilide exhibiting three proton singlet at 2.30 ppm in  $\text{CDCl}_3$  assigned to its methyl group, is employed as an internal standard. The two singlets at 4.2 and 4.05 ppm (in  $\text{CDCl}_3$ ) assigned to the 5- and 8-methoxy protons of khellin respectively, were chosen for its quantitative analysis (Fig.8). However, the presence of other ingredients in injectables interfere with the precise integration of the 5- and 8-methoxy signals. For this reason the 2-methylprotons singlet appearing at 2.4 ppm (in  $\text{CDCl}_3$ ) was used for assay of khellin in injectables. Ethanol-free chloroform was used as a solvent, as its proton singlet of 7.25 ppm does not interfere with upfield protons of both compounds. The method is rapid, accurate and precise, with standard deviations of  $\pm 0.76\%$  in synthetic mixtures and  $\pm 0.94\%$  in tablets and injectables respectively. No interference from tablet excipients could be observed. Visnagin shows in deuterated chloroform a very similar PMR spectrum to that of khellin, except for the presence of an aromatic proton singlet at 7.18 ppm and three proton singlet at 2.33 ppm assigned to its 8-H and 2- $\text{CH}_3$  group. This has allowed facile detection of visnagin in khellin in bulk drug and formulations. Moreover, the ratio determination of visnagin to khellin is achieved by integration measurements of the 2-methyl protons singlets at 2.33 and 2.40 ppm respectively (Fig.9).

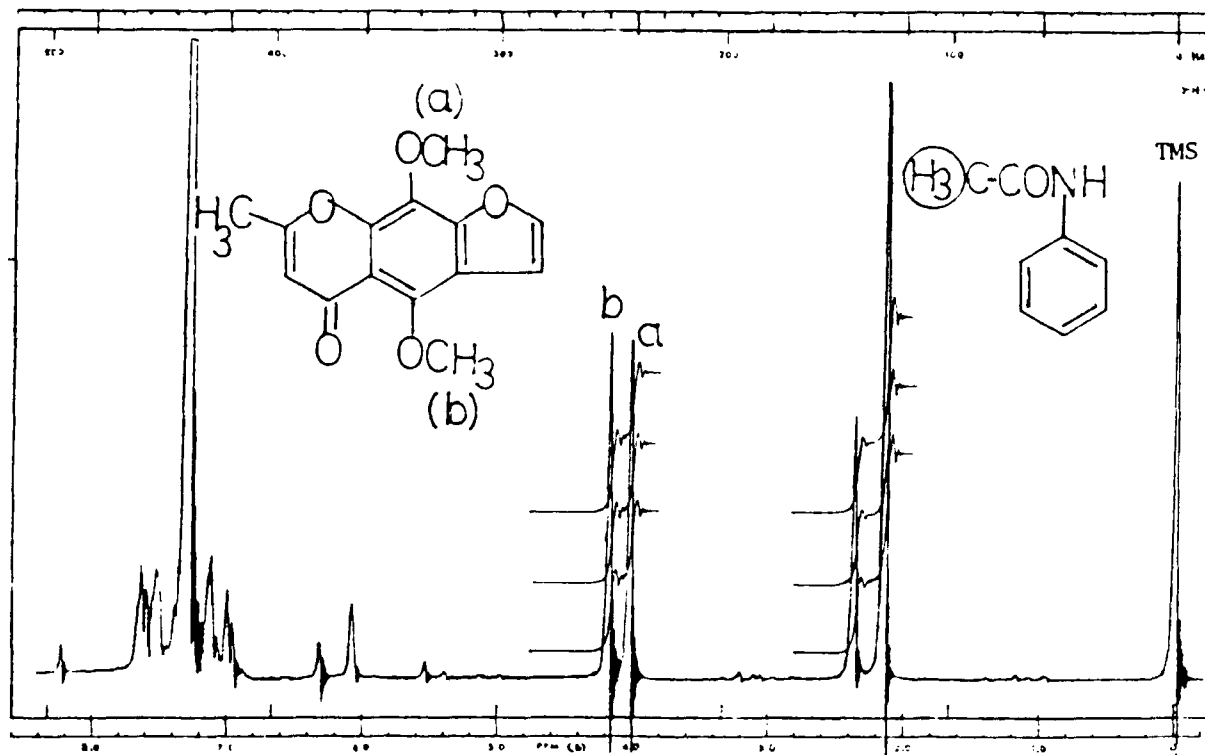


Fig. 8: PMR spectrum of khellin, acetanilide and TMS in ethanol-free chloroform.

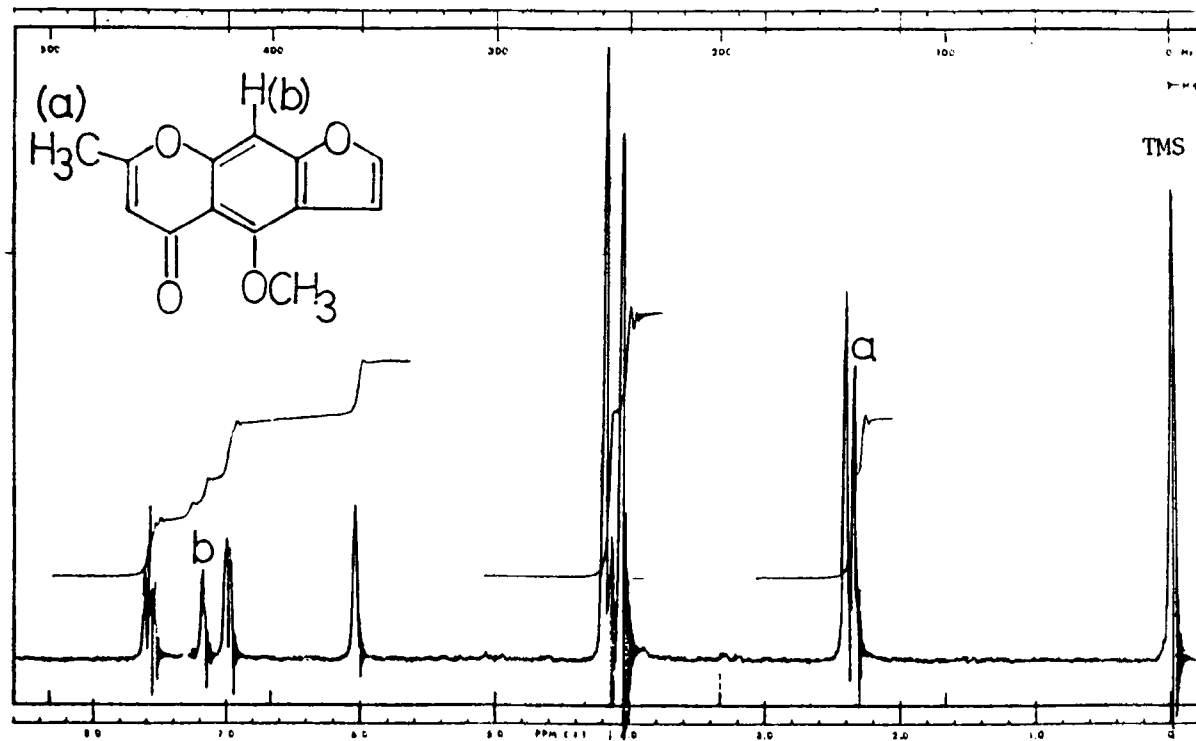


Fig. 9: PMR spectrum of khellin, visnagin and TMS in  $\text{CDCl}_3$ .

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# LORAZEPAM

*Jay G. Rutgers and Charles M. Shearer*

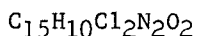
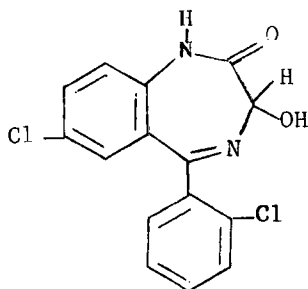
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## 1. Description

### 1.1 Name, Formula, Molecular Weight

The name used by Chemical Abstracts for lorazepam is 7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one.

The Chemical Abstracts Registry Number is 846-49-1.



Mol. Wt. = 321.2

### 1.2 Appearance, Color, Odor

Lorazepam is a white or nearly white, practically odorless, crystalline powder.

## 2. Physical Properties

### 2.1 Infrared Spectrum

An infrared absorption spectrum of a potassium bromide dispersion of lorazepam (Wyeth Reference Standard Lot C-10684) is presented in Figure 1. The spectral band assignments (1) are listed in Table I.

---

Table I

### Infrared Spectral Assignments of Lorazepam

<u>Wave number (cm<sup>-1</sup>)</u>	<u>Vibration Mode</u>
3500 to 2700	OH, NH stretch
1690	C=O stretch
1610	C=N stretch
1565 and 1475	Aromatic C=C stretch
825	Out of plane CH deformation of 1,2,4 substituted aromatic
730	Out of plane CH deformation of ortho disubstituted aromatic
750 and 695	1,2,4 substituted aromatic and ortho disubstituted aromatic. Unequivocal assignment cannot be made.

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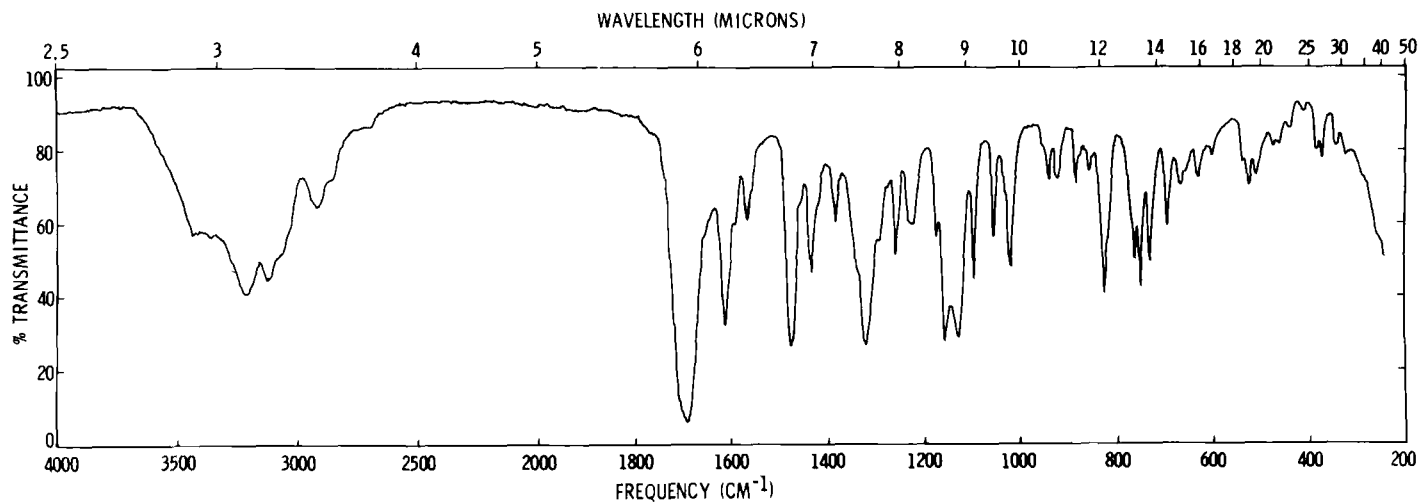


Figure 1 - Infrared Spectrum of Lorazepam (Wyeth Reference Standard, Lot C-10684) KBr pellet

## 2.2 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum sample (Wyeth Reference Standard, Lot C-10684) was prepared by dissolving 75 mg of it in 0.5 ml of deuterio-dimethylsulfoxide containing tetramethylsilane as internal reference. The spectrum was obtained on a 100 MHz Varian XL-100 spectrometer and is presented as Figure 2. Deuteration or irradiation of the OH reduces the methine doublet to a sharp singlet. The spectral assignments (2) are listed in Table II.

---

Table II

### NMR Spectral Assignments of Lorazepam

<u>Proton (No.)</u>	<u>Chemical Shift (ppm)</u>	<u>Type</u>	<u>J (in Hz)</u>
Aliphatic	4.88	Doublet	7.5
C-H(1)			
O-H(1)	6.38	Doublet	
Aromatic			
C-H(4)	7.4 to 7.7	Multiplet	
Aromatic			
C-H(1)	6.97	Doublet	2
Aromatic			
C-H(1)	7.6	Doublet of Doublet	
Aromatic			
C-H(1)	7.30	Doublet	9
N-H(1)	10.96	Broad singlet	

---

## 2.3 Ultraviolet Spectra

The ultraviolet spectrum of lorazepam in methanol is presented in Figure 3. The spectra of lorazepam in 1N NaOH and in 1N HCl are presented in Figure 4. The absorptivities and maximum wavelengths are given in Table III. These values agree with published data (3,4,5).

Levillain (6), has studied the relationship of structure and the UV absorption characteristics of a series of 1,4-benzodiazepines, including lorazepam, considering the electronic distribution of the various substitutes and the stereochemistry. The spectrum of lorazepam is consistent with that of other benzodiazepines with similar structure.

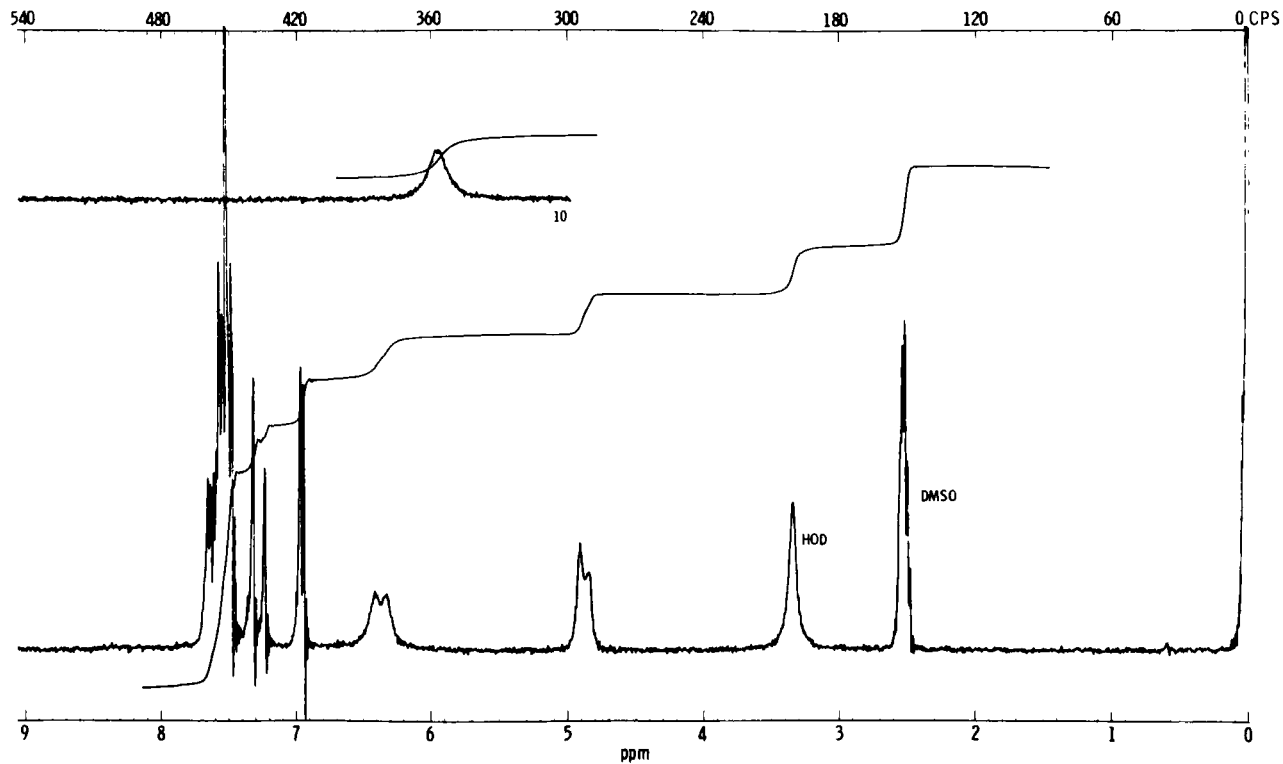


Figure 2 - NMR Spectrum of Lorazepam (Wyeth Reference Standard,  
Lot C-10684) in deuterio dimethylsulfoxide

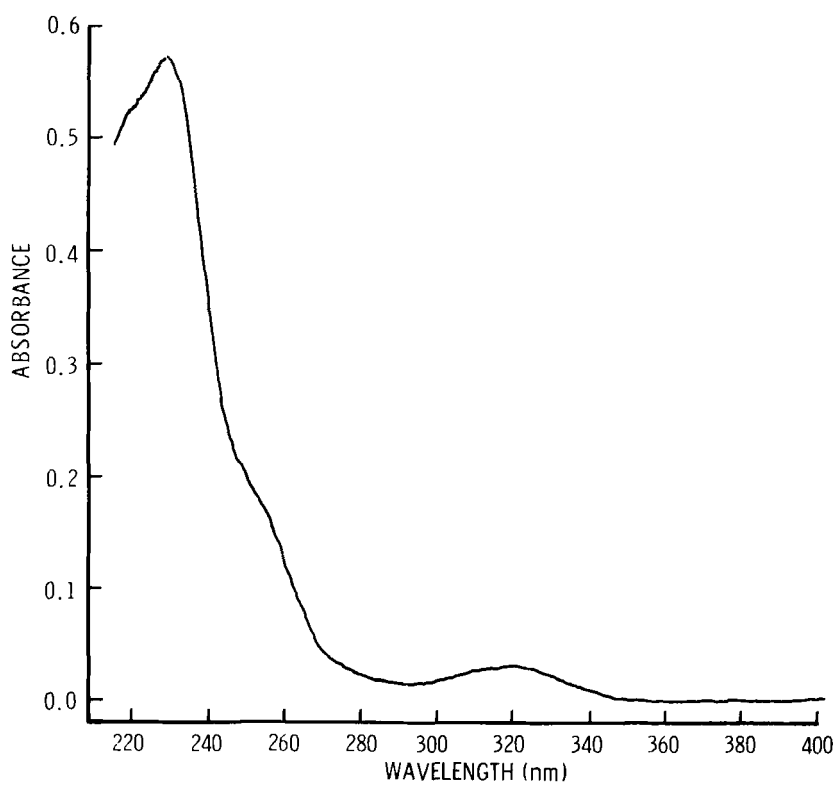


Figure 3 - Ultraviolet Spectrum of Lorazepam (Wyeth Reference Standard Lot C-10684) Solvent - methanol

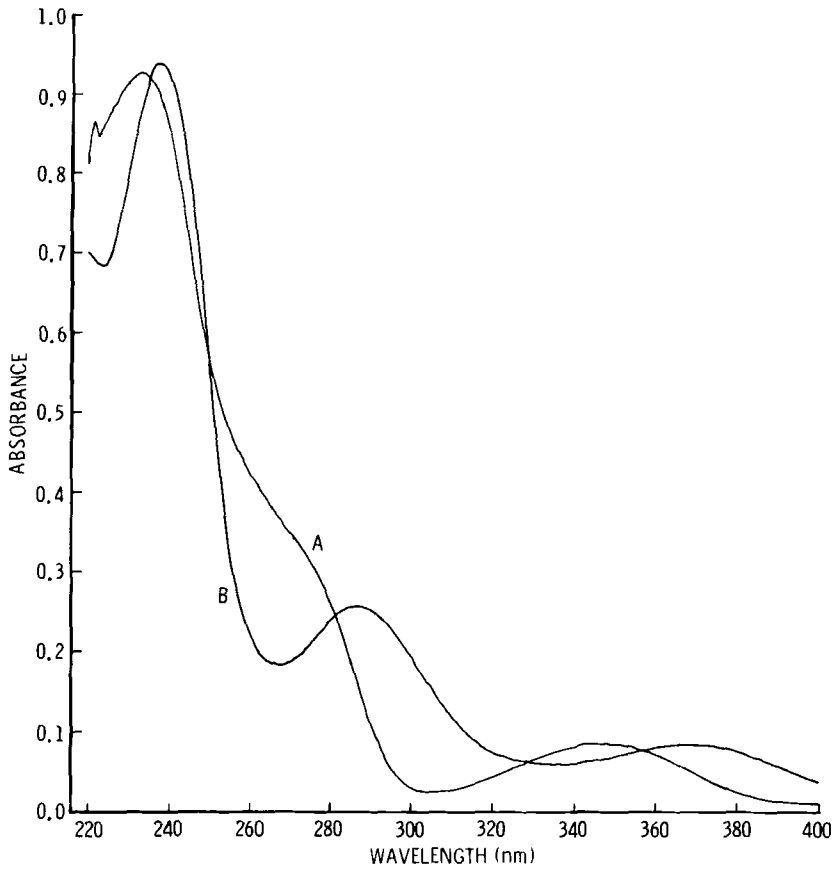


Figure 4 - Ultraviolet Spectra of Lorazepam (Wyeth Reference Standard, Lot C-10684) Solvent A, 1N NaOH; Solvent B, 1N HCl



Table III  
Ultraviolet Spectral Characteristics

<u>Solvent</u>	<u><math>\lambda</math> Max (nm)</u>	<u>Absorptivity</u>
methanol	320	6.1
	229	116
1N NaOH	347	8.4
	233	92
1N HCl	368	8.0
	287	25
	237	94

#### 2.4 Mass Spectra

The mass spectrum of lorazepam (Wyeth Reference Standard, Lot C-10684) was obtained with Kratos DS-50-S Data System coupled with a MS-902 double focusing, high resolution mass spectrometer (7). The ionizing electron beam energy was at 70 eV. Figure 5 is a bar graph of the mass spectrum with the molecular ion at m/e 320. Identification of the pertinent masses is presented in Table IV. A chemical ionization spectrum showed the parent peak at M+1, 321.

Table IV  
Mass Spectrum Fragmentation  
Pattern of Lorazepam

<u>m/e</u>	<u>Species</u>
320	M+
302	M+ - H <sub>2</sub> O
291	M+ - CHO
274	M+ - H <sub>2</sub> O - CO
263	M+ - HCO - CO
239	M+ - H <sub>2</sub> O - CO - Cl

#### 2.5 Melting Range

The following melting range temperatures have been reported.

<u>°C</u>	<u>Reference</u>
167 - 170 (d)	4
166 - 168 (d)	8

Trace amounts of certain acidic impurities, including 6-chloro-4-(o-chlorophenyl)-2-quinazolinecarboxylic acid (a possible degradation product), benzoic acid and salicylic acid, will markedly depress the decomposition temperature of lorazepam (9).

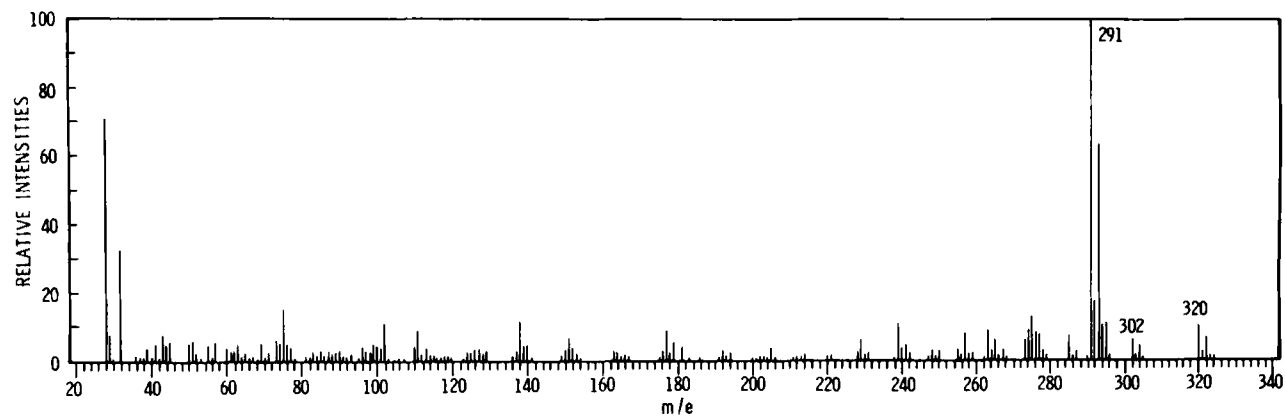


Figure 5 - Mass Spectrum of Lorazepam (Wyeth Reference Standard, Lot C-10684)

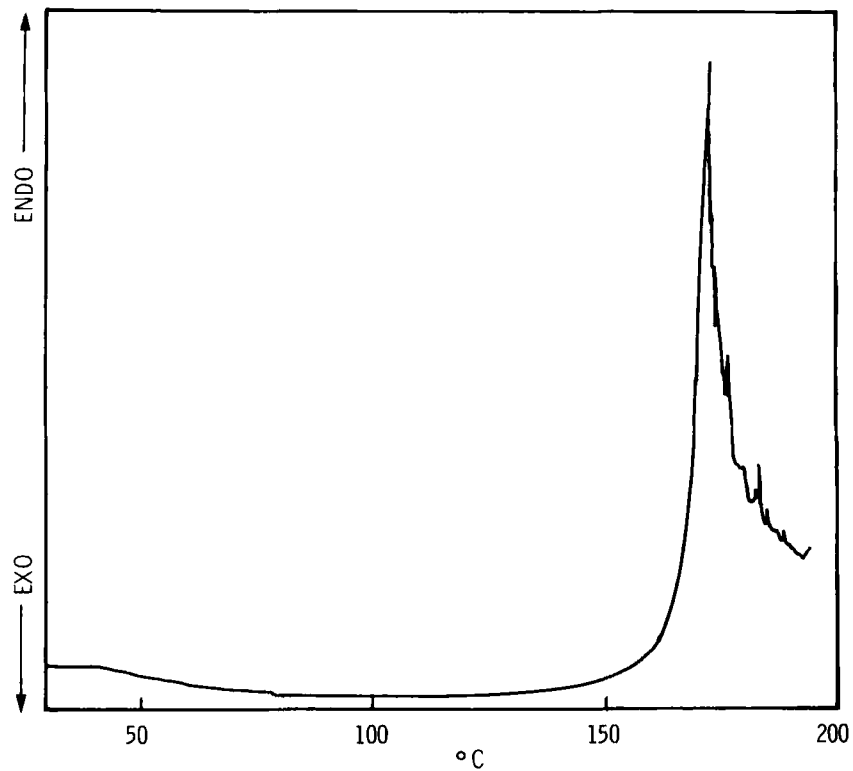


Figure 6 - Differential Thermal Analysis Spectrum of Lorazepam  
(Wyeth Reference Standard, Lot C-10684)

## 2.6 Differential Scanning Calorimetry

The DSC thermogram (10) of lorazepam (Wyeth Reference Standard, Lot C-10684) is shown in Figure 6. The thermogram was obtained at a heating rate of 10°C/min in a nitrogen atmosphere using a Perkin-Elmer DSC-2. The thermogram exhibits no endotherms or exotherms other than that associated with the decomposition melt.

## 2.7 Solubility

The following solubilities at room temperature have been reported.

<u>Solvent</u>	<u>Solubility (mg/ml)</u>	<u>Reference</u>
Alcohol	14	11
Water	0.08	11
Propylene glycol	16	12
Chloroform	3	13
Ethyl Acetate	30	13

## 2.8 Crystal Properties

The X-ray powder diffraction pattern of lorazepam (Wyeth Reference Standard, Lot C-10684), obtained (10) with a Phillips diffractometer using  $\text{CuK}\alpha$  radiation is presented in Figure 7. The calculated "d" spacings are presented in Table V. It is possible for lorazepam to form solvates and other crystal forms (9).

The crystal and molecular structure of the ethanol adduct of lorazepam have been characterized by X-ray analysis (14). The asymmetric unit consists of one ethanol and two lorazepam molecules linked together by hydrogen bonds. The crystals are monoclinic with cell dimensions of  $a=13.446\text{\AA}$ ,  $b=19.259\text{\AA}$  and  $c=13.789\text{\AA}$ . The  $\beta$  angle is  $116.80^\circ$ . The heterocyclic seven-membered ring adopts a boat configuration. The two phenyl rings are planar and the obtuse angles between them are  $106.6^\circ$  and  $99.1^\circ$ .

## 2.9 Dissociation Constants

Two  $\text{pK}_a$ 's are observed for lorazepam (15,5). The  $\text{pK}_a$  values, determined spectrophotometrically in aqueous buffers, are 1.3 and 11.5. Polarographically, the first  $\text{pK}_a$  was found (16) to be 1.8. Barret et al (5) have proposed that three species, a protonated, a neutral, and a deprotonated form, are involved in the equilibria. Protonation at low pH occurs at the nitrogen in the 4 position. Deprotonation occurs at high pH with the loss of the hydrogen atom from the 3-hydroxyl group.

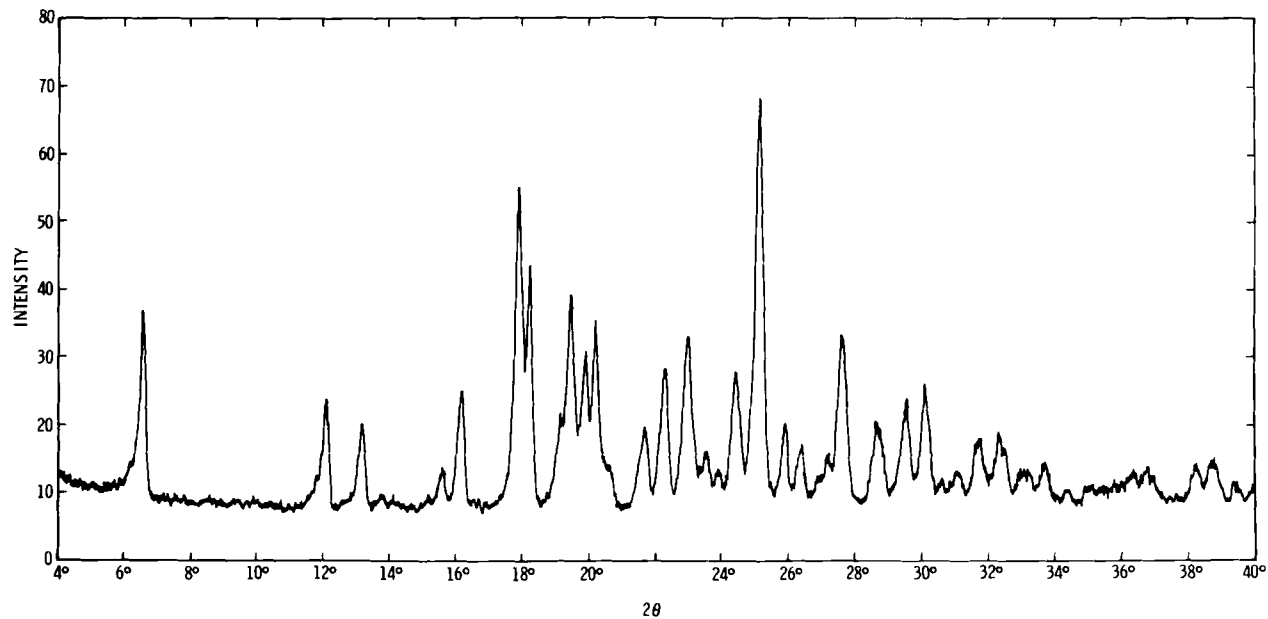


Figure 7 - X-Ray Diffraction Pattern of Lorazepam (Wyeth Reference Standard, Lot C-10684)

Hagel et al (17) have studied the nonaqueous titration of lorazepam with tetrabutylammonium hydroxide and perchloric acid. Their findings indicate that protonation does occur at the N-4 position. However, they propose that the deprotonation occurs at the N-1 position rather than at the 3-substituent.

---

Table V  
X-Ray Powder Diffraction Pattern

<u>d</u>	<u>I/I<sub>0</sub></u>	<u>d</u>	<u>I/I<sub>0</sub></u>
13.5	.46	3.65	.31
7.31	.26	3.54	1.00
6.71	.20	3.44	.18
5.68	.10	3.38	.13
5.47	.29	3.23	.40
4.96	.79	3.12	.20
4.86	.60	3.02	.23
4.56	.53	2.97	.29
4.46	.36	2.88	.07
4.40	.45	2.82	.15
4.10	.19	2.77	.17
3.99	.33	2.66	.10
3.87	.42	2.36	.10
3.83	.12	2.33	.11
3.72	.07		

---

### 2.10 Protein Binding

The protein binding of lorazepam and other benzodiazepines have been studied extensively by Mueller and Wollert (18-24) using circular dichroism and gel filtration techniques. The binding to albumin is decreased by the addition of chlorine in the ring 2' position as evidenced by the fact that oxazepam binding is greater than that of lorazepam. The binding is relatively independent of pH (pH 6.60 to 8.20).

### 3. Synthesis

One synthetic route for lorazepam is shown in Figure 8 beginning with 2-amino-2',5-dichlorobenzophenone (I). The benzophenone is first converted to its oxime (II) with

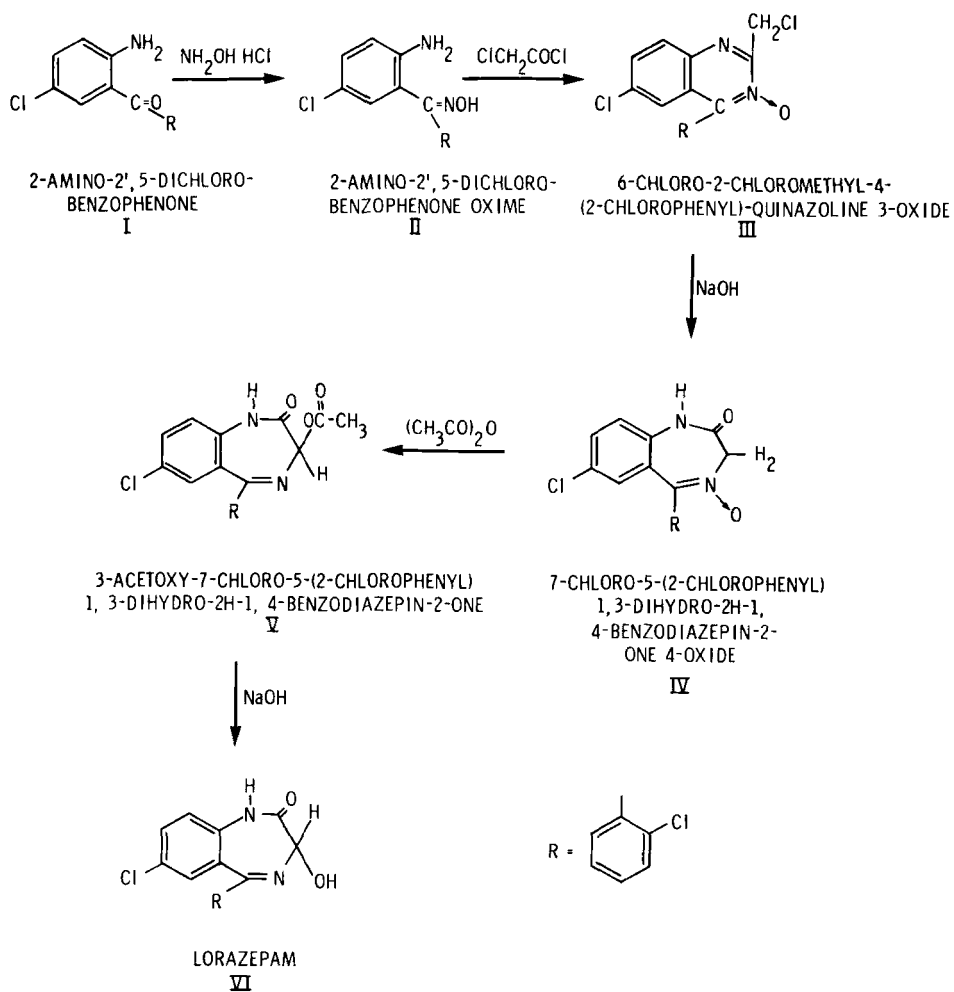


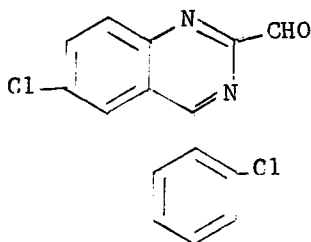
Figure 8 - Synthesis of Lorazepam

hydroxylamine. Reaction of the oxime with chloroacetyl chloride produces (III) the quinazoline 3-oxide (25). Ring enlargement to the benzodiazepin-2-one 4-oxide (IV) is accomplished by treatment with sodium hydroxide (26). Reaction with acetic anhydride and subsequent hydrolysis of the ester (V) with base produces (VI) lorazepam (8,27). A variation of this procedure is to react (IV) with isopropenyl acetate to form (V) which is hydrolyzed with base to produce lorazepam (28).

In another synthetic procedure the benzophenone anti-oxime is reacted with 2,2-diacetoxyacetyl chloride to produce a dihydroxyacetanilide derivative. This intermediate is cyclized with base and then hydrogenated to yield lorazepam (29).

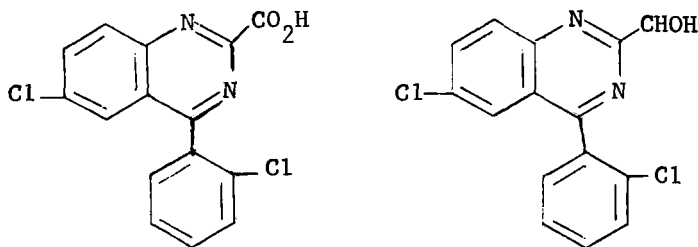
#### 4. Stability and Degradation

Lorazepam can lose a molecule of water and rearrange to form 6-chloro-4-(p-chlorophenyl)-2-quinazolinecarboxaldehyde (30).

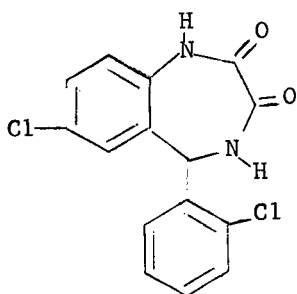


This quinazolinecarboxaldehyde can disproportionate and be oxidized or reduced to form the corresponding quinazoline-carboxylic acid or quinazoline alcohol respectively.





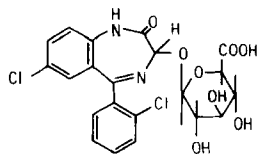
Acid hydrolysis of lorazepam ultimately produces 2-amino-2',5-dichlorobenzophenone which is the basis for numerous GLC, TLC and colorimetric analyses of lorazepam. In base lorazepam rearranges (31) to 7-chloro-5-(2-chlorophenyl)-4,5-dihydro-2H-1,4-benzodiazepin-2,3(1H)-dione.



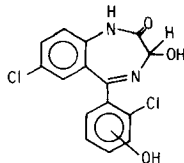
## 5. Metabolism and Pharmacokinetics

### 5.1 Metabolism

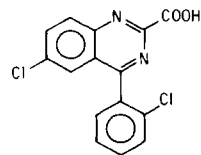
The metabolites of lorazepam which have been characterized in human and animal studies are shown in Figure 9. In man the major metabolite is the glucuronide (32,33,34). <sup>14</sup>C labeled studies have shown that 88% of the administered radioactivity was recovered in the urine and 7% in the stool. The glucuronide comprised 86% of the urinary activity. Minor metabolites are II,V,VI and VII. Characterization of the metabolites was made by mass spectrometry and by thin-



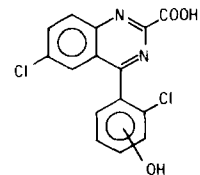
LORAZEPAM GLUCURONIDE (I)



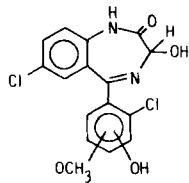
HYDROXYLORAZEPAM (II)



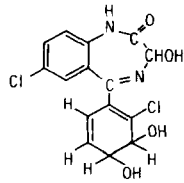
6-CHLORO-4-(4-CHLOROPHENYL)-  
2-QUINAZOLINECARBOXYLIC ACID (IV)



(VI)

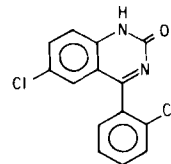


HYDROXYMETHOXYLORAZEPAM (III)

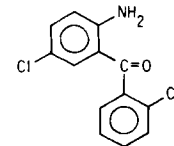


LORAZEPAM DIHYDRODIOL (IV)

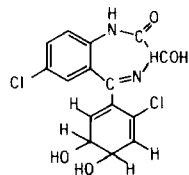
OR



6-CHLORO-4-(4-CHLOROPHENYL)-2(1H)-  
QUINAZOLINE (VII)

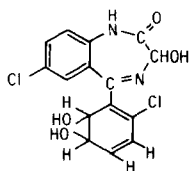


2-AMINO-2',5'-DICHLOROBENZOPHENONE (VIII)



LORAZEPAM DIHYDRODIOL (IV)

OR



LORAZEPAM DIHYDRODIOL (IV)

Figure 9- Metabolites of Lorazepam

layer chromatographic comparison to authentic samples. The glucuronide of lorazepam, in particular, has been characterized extensively by chemical analysis and infrared and mass spectroscopy of the trimethylsilyl derivative (35).

The glucuronide of lorazepam is also the major metabolite in miniature swine, dogs and cats (32,33,36,37). Minor metabolites in these species are II,III,V,VII and VIII. The metabolic transformation in rats is quite different from that in other animals investigated. Significant amounts of metabolite are found in plasma, bile and tissue. Compounds II,III,IV,V and VII have been identified as metabolic products (37).

A review of the metabolism of lorazepam has been written by Elliot (38).

### 5.2 Pharmacokinetics

The pharmacokinetics of lorazepam has been studied by Greenblatt et al. (33). A 2 mg oral dose of  $^{14}\text{C}$  lorazepam was administered to eight male adults. Blood samples were collected for a period of 96 hours and urine and feces samples 120 hours after administration. The various fractions were examined by means of a liquid scintillation spectrometer and gas chromatography. Data obtained on pooled plasma samples indicate that there is a lag time of about 35 minutes before the beginning of absorption. The apparent half-life of the absorption process is about 15 minutes for free lorazepam and 39 minutes for the glucuronide conjugate. Maximum plasma levels observed were 16.9 mg/ml for free lorazepam at 2 hours and 29.9 mg/ml for the conjugate at 4 hours. The apparent elimination half-lives are approximately 12 and 16 hours respectively. 88% of the total radioactivity administered was eventually recovered in the urine predominantly in the form of the conjugate. An additional 7% was recovered in the stool.

### 6. Identity

Kuhrent-Brandstaetter (4) has described several qualitative tests based on melting point or formation of color which can be used to identify lorazepam. A sample warmed in a phenylhydrazine solution forms crystals slowly when cooled. The crystals melt at 88-92°C. Upon continued heating the melt recrystallizes to orange-yellow crystals which remelt at

205-207°C. Heating a mixture of lorazepam and benzidine to the melting point produces an orange-brown melt.

A method has been developed for the detection of lorazepam in urine (39). A urine sample is extracted with ether and the ether extract examined under longwave UV light. A blue fluorescence due to the quinazolinone metabolite is indicative of lorazepam. The residue from the ether extract is then heated in 6N hydrochloric acid to produce the benzophenone derivative. A blue color developed with Bratton-Marshall reagent is also indicative of lorazepam. However, the test is not specific for lorazepam. Tetrazepam is reported to give the same positive tests.

Infrared spectroscopy can be used directly on the drug substance for its identification.

## 7. Methods of Analysis

### 7.1 Elemental Analysis

The elemental analysis of lorazepam (Wyeth Reference Standard, Lot C-10684) is presented below.

<u>Element</u>	<u>% Calculated</u>	<u>% Reported (7)</u>
C	56.10	56.05
H	3.14	2.99
N	8.72	8.65
Cl	22.08	21.77

### 7.2 Phase Solubility Analysis

Phase solubility analysis (9) on lorazepam (Wyeth Reference Standard, Lot C-10684) using isopropanol as the solvent gave a purity of  $99.8 \pm 0.2\%$ .

### 7.3 Direct Spectrophotometric Analysis

Seitzinger (3) has described an ultraviolet spectrophotometric method for the analysis of lorazepam in tablets. A sample equivalent to 5 mg of lorazepam is weighed into a 100-ml volumetric flask, 50 ml of alcohol is added and warmed in a steam bath. After cooling, the sample is diluted to volume with alcohol. The sample is filtered and a 10.0 ml aliquot of the filtrate diluted to 100 ml with alcohol. The absorbance is determined at 228 nm using alcohol as a blank and compared with the absorbance of a standard solution of lorazepam.

The adaption of the spectrophotometric method to automated analysis has been reported (40).

#### 7.4 Colorimetric Analysis

Lorazepam can be hydrolyzed with hydrochloric acid to form 2-amino-2',5-dichlorobenzophenone. The aromatic amine is diazotized with nitrous acid and the diazonium salt coupled to N-(1-naphthyl)ethylenediamine. The absorbance of the resulting colored solution is determined at 555 nm. A standard lorazepam solution is subjected to the same reactions for comparison. The procedure was applied to several tablet dosage forms of lorazepam. The tablets were extracted initially with chloroform and a portion of the chloroform extract evaporated for color development. Results obtained by the colorimetric procedure were in good agreement with those obtained by the spectrophotometric methods (3).

#### 7.5 Polarographic Analysis

Lorazepam is reducible at the dropping mercury electrode over a wide pH range. In the pH range of 0 to 6 a well defined wave is obtained. Above pH 6 the wave becomes strongly affected by absorption of the reducible species on the mercury electrode resulting in a distorted wave (41). The optimum pH range for analytical applications is considered to be 3 to 6. The diffusion current is linear with concentration in the range of  $10^{-5}$  to  $10^{-4}$ M (16).

Several analytical procedures for lorazepam tablet dosage forms employing methanolic acetate buffer (pH5) have been reported (16,42). The procedure can be adapted to differential pulse polarography (43,44,45). The polarographic technique has also been adapted to automated analysis by interfacing a polarographic analyzer with a continuous flow system (46,47). Polarographic analysis is stability indicating for the major route of degradation (30).

Oelschlager (48) has investigated the reduction of lorazepam and found that it consumes four electrons in three steps to form 7-chloro-5-(p-chlorophenyl)-1,3,4,5-tetrahydro-2H-1,4-benzodiazepin-2-one. The first step in the postulated mechanism is the reduction of the 4,5-N=C double bond with the consumption of two electrons. Water is eliminated with the formation of the aldimine. The aldimine is subsequently reduced with the consumption of two additional electrons.

### 7.6 Titrimetric Analysis

The tetrabutylammonium hydroxide titration procedure for oxazepam (NF XIV, 1975) was shown to be applicable to the titration of lorazepam. The titration is considered to proceed through the deprotonation at the N-1 position. Titration of lorazepam with perchloric acid in glacial acetic acid resulted in poorly defined potential breaks (17).

### 7.7 Chromatographic Analysis

#### 7.71 Thin-Layer Chromatography

Lorazepam may be evaluated on a thin-layer plate as the intact drug or, frequently, as the acid hydrolysis product, 2-chloro-2',5-dichlorobenzophenone. There are certain cases where it is advantageous to develop lorazepam as its hydrolysis product. Conversion to the benzophenone may be achieved by hydrolyzing in solution before spotting (49) or hydrolyzing directly on the plate after spotting (50). One method of detection is also based on conversion of lorazepam to the benzophenone after development (32).

The various solvent systems used for thin-layer chromatography of lorazepam on silica gel plates are given in Table VI. The table indicates those cases where the material was developed as the benzophenone. Table VII lists the methods of detection used for lorazepam on thin-layer chromatograms.

#### 7.72 Gas Chromatography

Gas chromatography has been used extensively in metabolic and pharmacological studies of lorazepam. This technique can provide the sensitivity which is required for the low doses usually administered.

Lorazepam is not thermally stable. A number of investigations (55-58) have shown that under gas chromatographic conditions lorazepam can lose a water molecule and rearrange to form 6-chloro-4-(2'-chlorophenylquinazoline)-2-carboxaldehyde. Consequently, in any gas chromatographic procedure where lorazepam is injected directly this rearrangement must be considered. Another consideration is that in metabolic studies the major metabolite is excreted as the glucuronide and a preliminary enzymatic incubation is usually employed. However, Marucci (59) was able to chromatograph the glucuronide directly by preparation of a derivative. The conjugate was first reacted with diazomethane to methylate the uronic acid carboxyl group and also the N position. The methyl derivative was then silylated with hexamethyldisilazane. The mass spectrum was consistent with a dimethyltrisilyl derivative. The procedure was

Table VI

Thin Layer Chromatography for Lorazepam

<u>Solvent</u>	<u>Rf x 100</u>	<u>Application</u>	<u>Reference</u>
Benzene	46(as benzophenone)	Separation of oxazepam and lorazepam	49
Chloroform-Acetone-Diethylamine (50:50:10)	Rm=80 (vs. meprobamate)	Identification of drugs in biological media	52
Hexane - 25% diethylamine in ethanol (75:25)	Rm=59 (vs. nitrazepam)	"	52
Toluene-acetone (80:20)(Tank contains ammonia vapor)	Rm=5 (vs. thioridazine)	"	52
Chloroform-ethanol-acetone (8:1:1)	33	Metabolic studies	32
Ethyl acetate-ethanol-conc. ammonia (5:5:1)	63	" "	32
Chloroform-methanol (10:1)	36	Identity in tablets	3
Benzene	41	Separation of 1,4-benzodiazepines	53
Heptane-chloroform-ethanol (50:50:5)	11	Separation of 1-4-benzodiazepines	51

Table VI (continued)

<u>Solvent</u>	<u>Rf x 100</u>	<u>Application</u>	<u>Reference</u>
Ethyl acetate - 1,2-dichloroethane - 25% ammonium hydroxide (80:20:5)	25	Separation of 1-4-benzodiazepines	51
Ethyl acetate-ethanol-25% ammonium hydroxide (50:50:10)	61	"	51
Heptane-chloroform-ethanol (50:50:10)	24	"	51
Ethyl acetate - 1,2-dichloroethane - 25% ammonium hydroxide (80:20:10)	20	"	51
Cyclohexane-ethyl acetate-ethanol - 25% ammonia (20:20:7:0.1)	42 (as benzophenone)	Identification of 1,4-benzodiazepines in urine	54



Table VIITLC Detection Methods for Lorazepam

<u>Method</u>	<u>Color</u>	<u>Detection Limit (μg)</u>	<u>Reference</u>
Quenching of Phosphorescence on a phosphor- escent plate under shortwave UV light	Dark spot against a green background	0.1	3
Conc. HCl spray, heat, followed by Bratton-Marshall spray	Blue-violet	0.02	3
Conc. H <sub>2</sub> SO <sub>4</sub> spray, observe under longwave UV light	Green	0.01	51
Mercuric chloride- diphenylamine spray	Blue	NA*	52

\* Data not available

applied to glucuronide levels in urine from humans and animals.

An alternate technique is conversion of lorazepam to its benzophenone derivative prior to injection. An example of this is the procedure developed by Knowles et al. (60) for determination of free and conjugated lorazepam in serum, urine and feces. The biological sample is adjusted to pH 7 and extracted with ether to remove free lorazepam. The aqueous phase is adjusted to pH 4.5 and incubated overnight with  $\beta$ -glucuronidase to cleave the conjugate. The aqueous phase is again adjusted to pH 7 and extracted with ether. Lorazepam is re-extracted into 12N sulfuric acid and then heated at 100° to form the benzophenone. The samples are dissolved in toluene prior to analysis. The limit of detection was 0.01 ug/ml.

The conditions for various methods are given in Table VIII.

#### 7.73 High Performance Liquid Chromatography

Gonnet (64) has developed an isocratic elution technique for separating lorazepam from a series of other benzodiazepines (medazepam, diazepam, nitrazepam, chloazepate, oxazepam and chlordiazepoxide). Separations were achieved on a 20 cm x 4.6 mm column packed with Lichrosorb SI 60, 5 micron, at a pressure of 1090 psi and a flow rate of 2.6 ml/min. The mobile phase was dichloromethane-isopropanol (96:4) saturated with water.

de Silva (65) discusses the high performance liquid chromatography of lorazepam and other benzodiazepines. A reverse phase column ( $\mu$  Bondapak C-18) was used with an eluant consisting of methanol (500 ml); isopropanol (50 ml); pH 3.25 1M potassium phosphate buffer (1 ml); diluted to 1000 ml with distilled, deionized water. A normal phase system (10  $\mu$  Partisil silica gel) had an eluant of methylene chloride (95):methanol (5). The reverse phase column was the better.

Lorazepam can be separated (12) from its degradation products (see Section 4) by an eluant consisting of acidic aqueous acetonitrile on a reverse phase column.

High performance liquid chromatography has been used to analyze for benzodiazepines including lorazepam in tissue (66). An eluant of 60% methanol (v/v) in phosphate buffer (pH 7.8) eluted lorazepam in 4.2 ml from a column of Spherosorb-5-ODS (150 mm x 4.6 mm id).

Table VIIIGas Chromatographic Systems for Lorazepam

<u>Injected As</u>	<u>Column Packing</u>	<u>Col- umn</u>	<u>Carrier Gas</u>	<u>Column Temp.</u>	<u>Detec- tor</u>	<u>Ref.</u>
Lorazepam	3% OV17 on Gas Chrom Q	1m x 2mm glass	Nitrogen at 42 ml/min	255°C	Electron capture	55
Lorazepam	3% OV17 on Gas Chrom Q (60/80)	4 ft x 4mm borosili- cate glass	Argon-methane (90:10) 120 ml/min	240°C	Electron capture	61
Lorazepam	3% OV17 on Gas Chrom Q (100-120)	2m x 2mm	Helium 30 ml/min	280°C	Mass Spectro- meter	56
Lorazepam	3% OV17 on Gas Chrom Q (60/80)	4 ft x 4mm borosili- cate glass	Argon-methane (90:10) 75 ml/min	230, 210°C	Electron capture	62
Trimethyl- silyl deri- vative of Lorazepam	3% OV17 on Gas Chrom Q (100-120)	4 ft x 4mm borosili- cate glass	Argon-methane (90:10) 75 ml/min	210°C	Electron capture	62

Table VIII (continued)

<u>Injected</u> <u>As</u>	<u>Column</u> <u>Packing</u>	<u>Col-</u> <u>umn</u>	<u>Carrier</u> <u>Gas</u>	<u>Column</u> <u>Temp.</u>	<u>Detect-</u> <u>tor</u>	<u>Ref.</u>
Methyltri- methyl- silyl deri- vative of Lorazepam glucuronide	3% OV17 on Gas Chrom Q (100-120)	1m glass	Nitrogen 30 ml/min	320°C	Flame ionization	59
Benzo- phenone	3% OV17 on Chromosorb W (80/100)	10 ft x 2mm stain- less steel	Helium 20 ml/min	280°C	Electron capture	60
Benzo- phenone	SE 30	2m glass	Nitrogen 40 ml/min	240°C	Flame ionization	53
Benzo- phenone	6% QF-1 on Anakrom ABS (80/90)	6 ft x 1/8" stainless steel	Helium 40 ml/min	240°C	Electron capture	34
Lorazepam	3% OV-7 on Varaport 30 80/100	2m x 4mm glass	Argon 80 ml/min	250°C	Electron capture	63

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# METHOXSALEN

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## 1. Description

### 1.1 Nomenclature

#### 1.11 Chemical Names

9-Methoxy-7H-furo [3,3-g]  
[1] benzopyran-7-one;  $\delta$ -Lactone  
of 6-hydroxy-7-methoxy-5-benzo-  
furanacrylic acid; 8-Methoxy  
[furano-3',2': 6,7 - coumarin];  
8-Methoxy-4, 5': 6,7-furano-  
coumarin.

#### 1.12 Generic Names

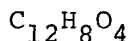
Ammoidin, Xanthotoxin, 8-Methoxy-  
psoralen, Methoxsalen.

#### 1.13 Trade Names

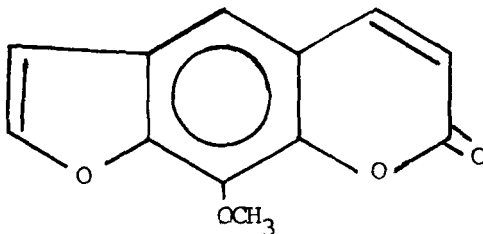
Meladinin, Meloxine, 8-MOP, 8-MP,  
Methoxa-Dome, New-Meladinin,  
Oxsoralen.

### 1.2 Formulae

#### 1.21 Empirical



#### 1.22 Structural



1.23 Wiswesser Line Notation

T C566 DO LV OJ BOI

1.3 Molecular Weight

216.18

1.4 Elemental Composition

C, 66.67%; H, 3.73%; O, 29.60%.

1.5 Appearance, Color, Taste, Odor

Silky fluffy needles or long rhombic prisms, white to cream-colored, bitter taste followed by tingling sensation, odorless.

2. Physical Properties2.1 Crystal Properties2.11 X-Ray Diffraction

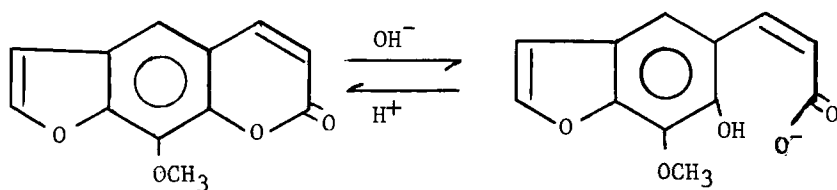
The crystallographic properties of methoxsalen has been reported(1).

2.12 Melting Point

Methoxsalen melts between 143 and 148°(2).

2.2 Solubility

Methoxsalen is practically insoluble in cold water, sparingly soluble in boiling water and freely soluble in chloroform. It is soluble in boiling alcohol, acetone, and acetic acid. It is also soluble in aqueous alkalis with ring cleavage, reconstitution occurs upon neutralisation.



### 2.3 Identification

- a- An alcoholic solution of methoxsalen gives a deep-violet color with 8-amino-5-hydroxy-2-methyl-furo-4', 5', 6, 7 - chromone, in the presence of alkali (3). The test is sensitive to 0.02 mg of the drug.
- b- The UV absorption spectrum of a 1 in 125,000 solution in alcohol exhibits maxima and minima at the same wavelengths as that of a similar solution of USP Methoxsalen Reference Standard, concomitantly measured (4).
- c- Dissolve, by heating, about 10 mg in 5 ml of diluted nitric acid, the solution turns yellow. Render the solution alkaline with sodium hydroxide, the solution turns brown (4).

### 2.4 Spectral Properties

#### 2.41 Ultraviolet Spectrum

Methoxsalen exhibits a characteristic UV Spectrum (Fig.1), in 95% ethanol, with the following electronic absorption bands (5):

$\lambda_{\text{Max}}$	Log $\epsilon$	$\lambda_{\text{min}}$	Log $\epsilon$
219	4.48	232	4.23
245	4.44s.	262	4.23s.
249	4.46	276	3.90
262	4.23s.		
301	4.16		

s. = Shoulder

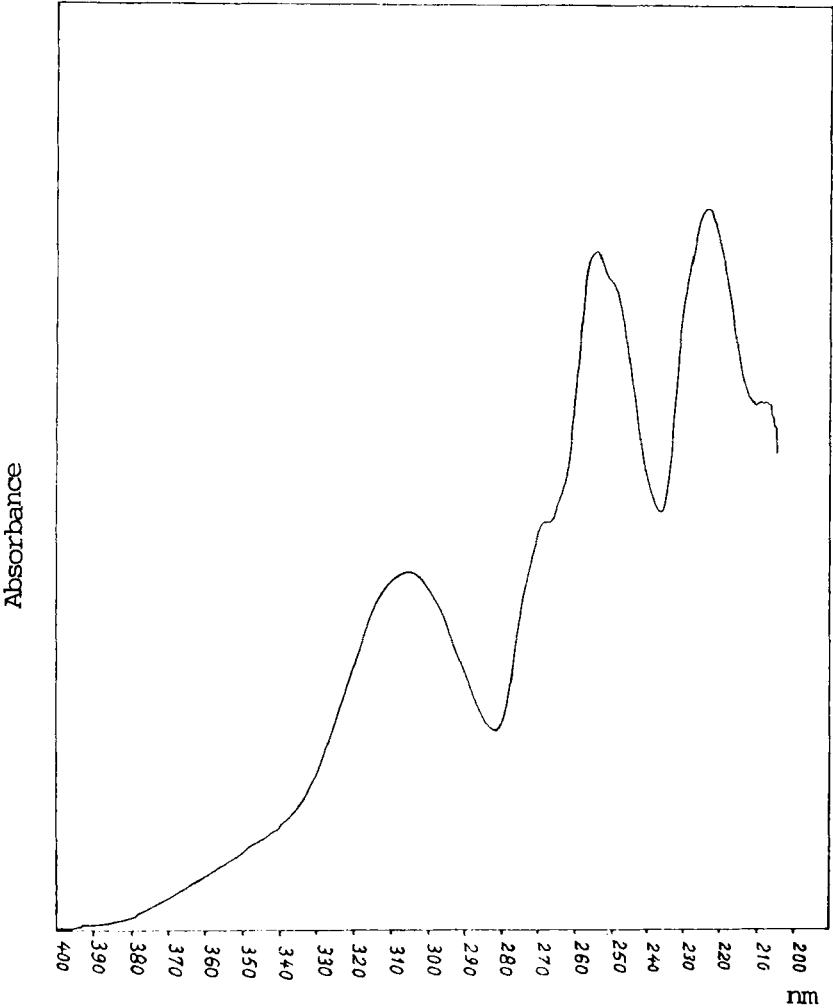


Fig. 1 - UV spectrum of Methoxsalen in ethanol

Other UV spectral data for methoxsalen and other psoralens have been reported (6-9).

#### 2.42 Infrared Spectrum

The IR spectrum of methoxsalen has been determined in nujol on a Unicam SP - 200 (Fig.2). The structural assignments have been correlated with the following band frequencies:

Frequency $\text{Cm}^{-1}$	Assignment
3110, 3080, 3040	CH
1705	C=O( $\alpha$ -pyrone)
1620	C=C(aromatic and $\alpha$ -pyrone)
1580, 1540	C=C(aromatic)
1150	C-O-C
875	Furan ring
800	Isolated H (penta substituted aromatic)

These findings are in agreement with reported data (5,9). Other finger print bands characteristic of methoxsalen are: 1400, 1380, 1340, 1300, 1220, 1180, 1120, 1100, 1020, 1000, 820 and  $760 \text{ cm}^{-1}$ .

#### 2.43 Nuclear Magnetic Resonance Spectrum

The proton magnetic resonance spectra of methoxsalen and other furocoumarins have been investigated (5,9,10,11). A typical PMR spectrum of methoxsalen is shown in Fig.3. The sample was dissolved in  $\text{CDCl}_3$  and the spectrum was recorded on a T60A NMR spectrometer, using tetramethylsilane as a reference standard.

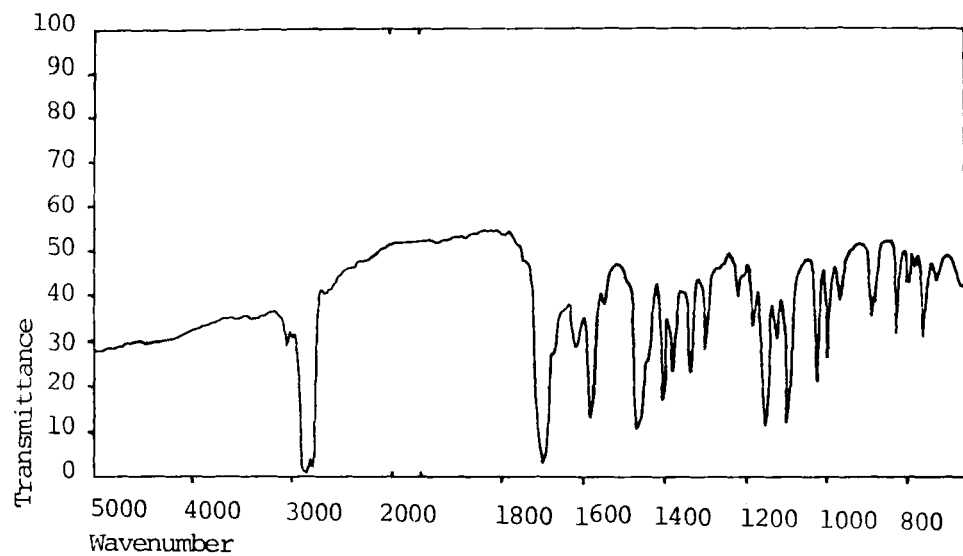


Fig. 2 - IR Spectrum of Methoxsalen in nujol.

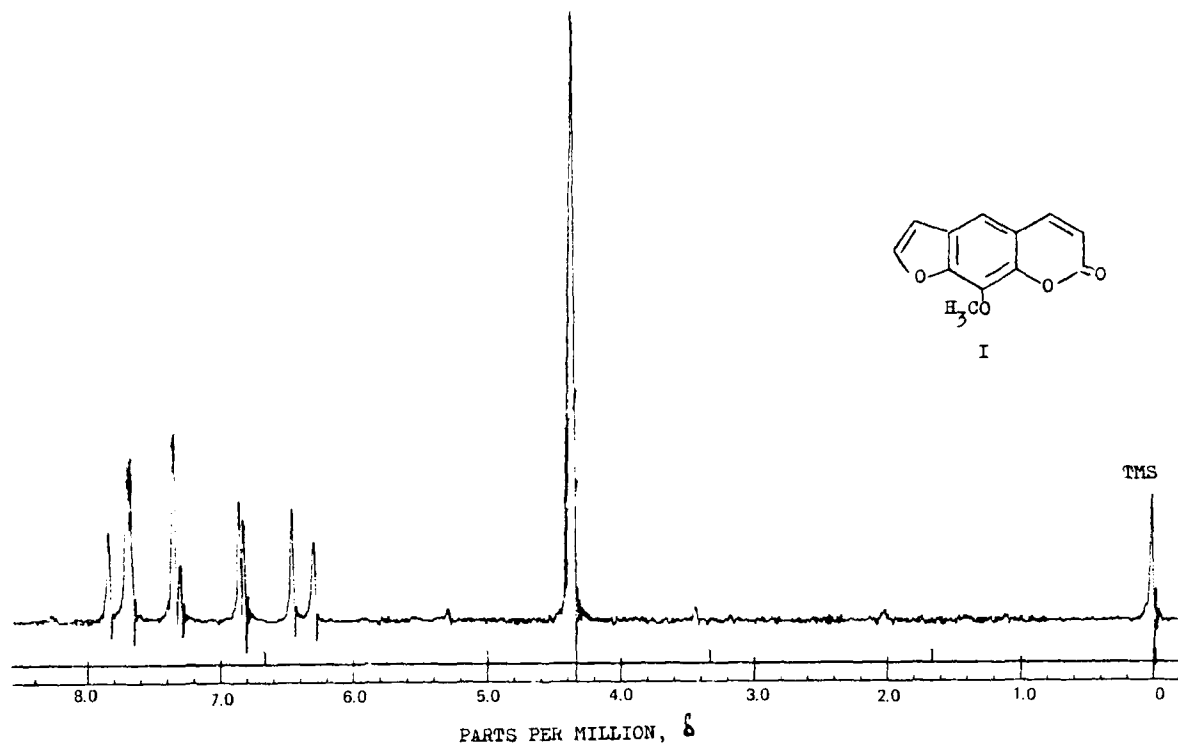


Fig. 3 - PMR spectrum of Methoxsalen and TMS, tetramethylsilane in deuterated chloroform.

The PMR data of methoxsalen and its biologically inactive isomer, bergapten (5-methoxypsoralen) are illustrated in table I.

Table I: PMR characteristic of Methoxsalen and Bergapten.

	Chemical shifts ( $\delta$ )							
	5-OCH <sub>3</sub> s <sup>a</sup> 3	8-OCH <sub>3</sub> s 3	3-H <sub>b</sub> d <sup>b</sup>	4-H d	5-H s	8-H d	4'-H d	5'-H d
Methoxsalen	-	4.35	6.38	7.75	7.33	-	6.83	7.66
Bergapten	4.25	-	6.23	8.10	-	7.25	7.03	7.53

a<sub>s</sub> = Singlet.    b<sub>d</sub> = Doublet.

Also other PMR studies on methoxsalen and analogues have been published (5,9,10).

#### 2.44 Mass Spectrum

The mass spectrum of methoxsalen, obtained by conventional electron-impact ionization, shows a molecular ion M<sup>+</sup> at m/e 216.04 (12,13). The M<sup>+</sup> ion peak is the base peak (Fig.4). The fragmentation patterns of methoxsalen and other psoralen derivatives have been reported (5,13,14).

### 3. Isolation

Fahmy and Abu-Shady (15-17) have reported the isolation of methoxsalen from the fruits of Egyptian, umbelliferous Ammi majus (L) plant. The dried powdered Ammi majus (L) (Fam.



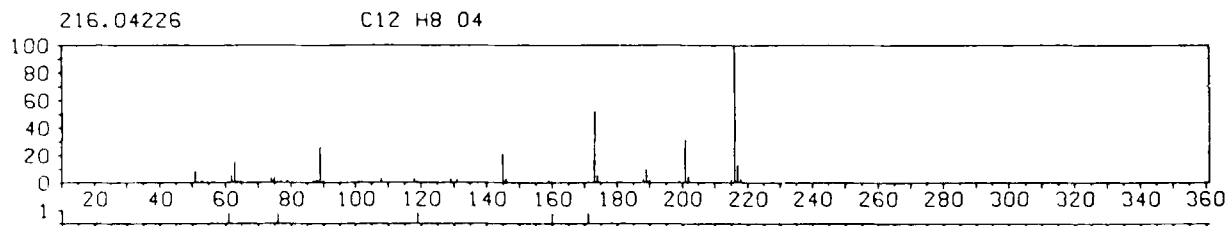


Fig. 4 - Mass Spectrum of Methoxsalen

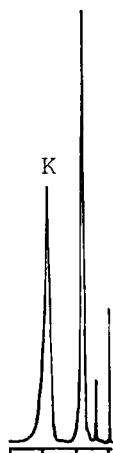


Fig. 8 - A typical Chromatogram of Methoxsalen and Khellin in methanol-water mixture (60:40).

M = Methoxsalen  
K = Khellin

umbelliferae) fruits are exhaustively extracted with petroleum ether (60-80°). The deep green extract is concentrated and kept for overnight and the greenish resinous crystalline deposit is filtered and crystallised from ethanol. The yield is 0.25%. The isolation of methoxsalen and other furocoumarins have been also described by other co-workers (18-26).

#### 4. Biosynthesis

The exact biogenetic pathway leading to the formation of methoxsalen is uncertain. One of the problems still to be resolved concerns the mechanism by which linear furocoumarins are formed from their 2', 3'-dihydro-2'- isopropyl counterparts (27). It seems certain that marmesin is directly involved and tracer experiments indicate that (+) -(S)- form, rather than the (-) - (R) - (nodakenetin), is preferentially incorporated (28,29). The sequence of further substitution of furanocoumarins has been studied by Caporale, et al (30, 31). The most recent suggestion is that further substitution, hydroxylation followed by methylation, occurs after furan ring formation. Scheme I illustrates the formation of methoxsalen starting from trans-cinnamic acid.

#### 5. Synthesis

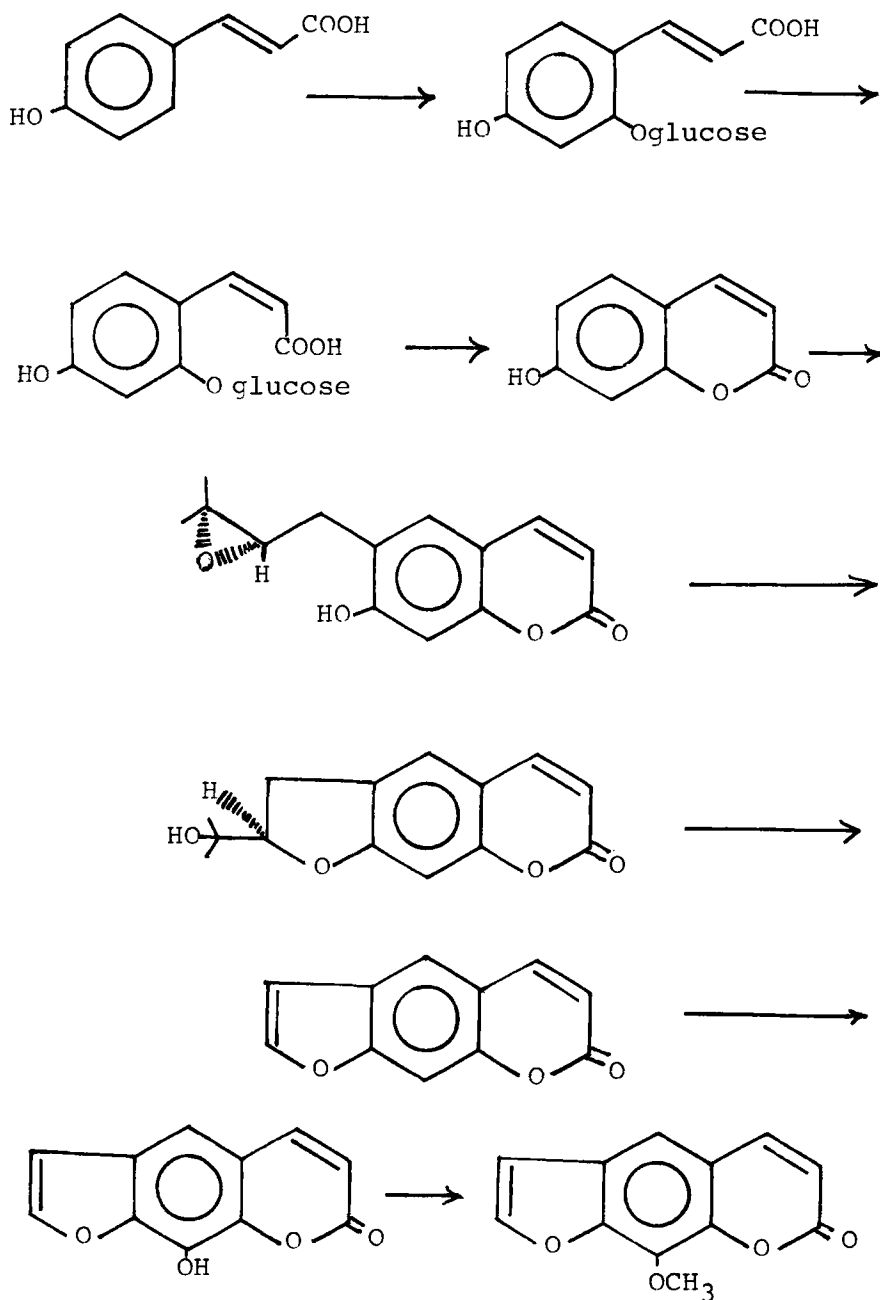
The synthesis of methoxsalen has been achieved by two main routes.

Route I: Benzofuran route (32-34), which involves the hydrogenation of 6,7-dihydroxybenzofuran (I) or 6,7-dihydroxycoumaran-3-one (II) to afford 6,7-dihydroxycoumaran (III). III was then converted, by Pechmann reaction with malic acid, to 8-hydroxy-4',5'-dihydro-6,7,3',2'-furanocoumarin (IV). Methylation of IV followed by dehydrogenation gives methoxsalen (V).

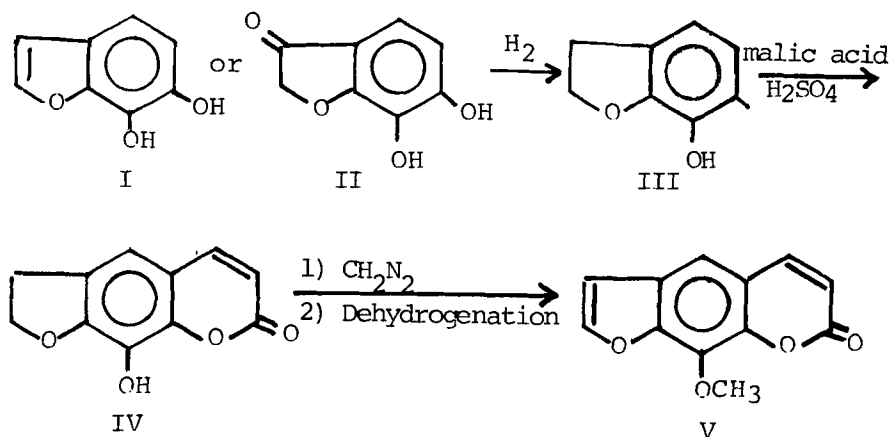
Route II: Umbelliferone route (35), involves the use of a coumarin ring instead of a benzofuran derivative. Although it is an improvement on the previous route, it gives a low

## Scheme I.

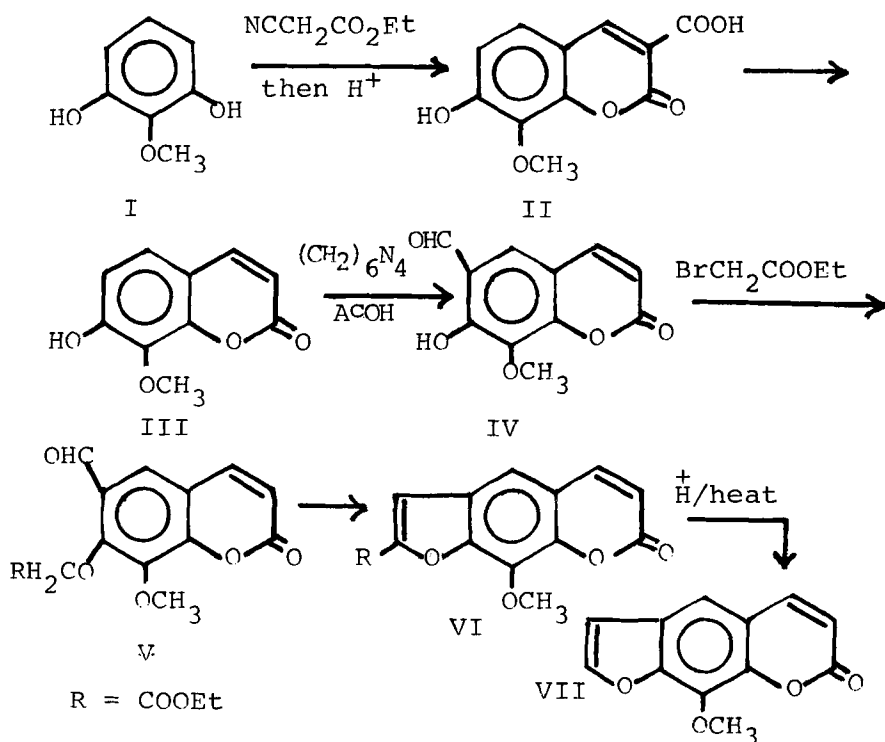
Biosynthesis of methoxsalen from trans-cinnamic acid.



## Route I (Benzofuran route):



## Route II (Umbelliferone route):



yield (33%). In this route, 7-hydroxy-8-methoxycoumarin (III) is the key intermediate for the synthesis of methoxsalen. III is converted into the 6-formyl derivative which in turn is cyclised, using ethyl bromoacetate to effect ring closure, to VII. This approach is attractive in that an umbelliferone is an intermediate rather than 6-hydroxycoumaran. Umbelliferones can be more easily synthesised and are more readily available from natural sources.

Other methods for the synthesis of methoxsalen and analogues are also reported (19,36-39).

## 6. Metabolism

Very little is known about the metabolism of photosensitising furocoumarins (40). By contrast no data are so far available about the metabolism of methoxsalen. However studies have been performed in mice and human volunteers on the absorption, metabolism and excretion of psoralen and trimethylpsoralen (41,42).

## 7. Methods of Analysis

### 7.1. Colorimetry

Colorimetric determination of methoxsalen in the Ammi majus fruits and tablet formulation has been described (43,44). The method involves the addition of 0.5M potassium hydroxide solution to the drug, after 30 minutes, diazotized sulfanilic acid solution (a 1:1 - mixture of 0.64% sulfanilic acid solution in dilute hydrochloric acid and 0.4% aqueous sodium nitrite) is added. The extinction of the solution is then measured and referred to a calibration curve prepared by a standard methoxsalen solution. Schonberg, et al (3) have described another colorimetric procedure for the determination of methoxsalen and other psoralens. The method involves the addition of 1 ml of a 0.1% solution of 8-amino-5-hydroxy-2-methylfuro (4',5',6,7) chromone to 2 ml of an alcoholic solution of the psoralen. The reaction

mixture is made alkaline with 10 ml of a buffer solution (pH 11.6), a violet color is gradually developed and can be measured.

## 7.2 Spectrophotometry

Methoxsalen and bergapten have been determined in the plant material, by measuring the absorbance of the chloroform extract at 300 and 311 nm, respectively (45). Fedorin and Georgrievskii (46) have described a spectrophotometric procedure for the estimation of methoxsalen and bergapten in Beroxan preparations. Yeagers and Augenstein (47) have described the absorption and emission spectra of methoxsalen and psoralen in powders and in solutions.

Chakrabarti, et al (48) have developed a spectrophotometric method for the estimation of methoxsalen in the plasma. The method is based on extraction of the plasma, acidified with hydrochloric acid, with benzene-ethyl acetate mixture (9:1) and the solvent is evaporated, to dryness. The residue is dissolved in xylene and the absorbance is measured at 300 nm. The drug extracted from plasma is characterised by TLC, UV absorption spectrum and GC/MS fragmentation pattern. The U.S.P. method for the assay of methoxsalen is spectrophotometric (4) one.

## 7.3 Fluorimetry

The assay of methoxsalen and bergapten in Beroxan preparations has been achieved fluorimetrically (46).

## 7.4 TLC - Fluorimetry

Chakrabarti, et al (49) have described a rapid and sensitive method for the determination of methoxsalen in the plasma. The plasma samples are acidified with hydrochloric acid and heated in a boiling water-bath to release the plasma-

bound drug. The drug is extracted by a solvent system (benzene-ethylacetate, 9:1). The extract is evaporated to dryness and the residue is dissolved in methylene chloride and is spotted on thin layer plates and developed with the same solvent system. The plates are visualized under UV light (320-400 nm) and scanned. The method is sensitive up to 20 ng of methoxsalen. The overall recovery of the drug from the plasma is 84%. The identity of the recovered drug was confirmed by GC/MS (Fig.5).

#### 7.5 Time-resolved phosphorimetry

Phosphorescence spectra for methoxsalen and psoralen have been recorded for 300°K and 77°K. The peaks for 300° fluorescence excitation spectra obtained from a "front face" cell agreed with peaks in the absorption spectra, when correlations have been made for the output of the exciting lamp. At 77°K the phosphorescence lifetimes vary from 0.4 to 1.1 seconds (47).

#### 7.6 Electrophoresis

Berlingozzi and Parrini (50) have described a method for separation of methoxsalen from other coumarin derivatives by circular paper electrophoresis. The compounds are first subjected to circular paper chromatography in water-acetic acid-butylene glycol (86:10:6). The  $R_f$  values found in strips, complete circle, and a 90° sector of circle, and the color of fluorescence in Wood's light are, respectively 0.602, 0.779 and 0.786, light green colour for methoxsalen. The electrophoretic experiments are conducted in a buffer (pH9) of sodium barbital, sodium acetate, potassium oxalate, 0.1 N hydrochloric acid, and water. The travelling distances for methoxsalen and other coumarins are reported. A better separation has been obtained by means of electrophoresis than chromatography.

## 7.7. Chromatography

### 7.71 Paper chromatography

Methoxsalen and other psoralens have been separated by paper chromatography. The following solvent systems have been used, in a unidimensional ascending method (51): water; water-methylethylketone (17:3); water-ethanol-methylethylketone (15:3:2); water-formamide-methylethyl ketone (9:3:2)); formamide-ethylacetate-water (8:5:3) and butanol-acetic-acid-water (4:1:1).

Grujić-Vasić (52) has described a paper chromatographic separation of methoxsalen and some psoralens, using the following solvent systems: water-saturated ammonium hydroxide; butanol-ethanol-concentrated ammonium hydroxide-water (4:4:1:1); and propanol-water (90:10, 80:20, 70:30, and 20:80).

Beyrich (45) has reported a method for the quantitative determination of methoxsalen, bergapten, and imperatorin in the dried plant material. The powdered plant is extracted with chloroform in a Soxhlet, the extract is evaporated to dryness and the residue is dissolved in toluene. An aliquot (10-100  $\mu$ l) is applied to a paper impregnated with dimethylformamide and developed with heptane-benzene mixture (4:2). The separated methoxsalen is eluted with chloroform and determined spectrophotometrically. Heptane-benzene mixture (4:1 and 7:3) has been also used (53,54) on paper impregnated with formamide, for the detection of methoxsalen and other



psoralens. The detection has been achieved by their fluorescence alone, and after treatment with 0.5N ethanolic potassium hydroxide, by the diazoreaction, and by the use of Emerson phenazone-potassium ferricyanide reagent.

Lutowski, et al (43) have described a paper chromatographic method for quantitation of methoxsalen in Ammi majus fruits and their preparations. The powdered sample is extracted with methanol in a Soxhlet and the extract is applied to Whatman No.1 paper, previously imprgnated with dimethylformamide-ethanol mixture (2:3). The chromatogram is then developed with heptane-benzene (7:3) by the descending-solvent technique. The spot of methoxsalen is eluted with ethanol and then determined colorimetrically.

#### 7.72 Thin Layer Chromatography

The isolation and detection of methoxsalen from other coumarins, by TLC methods, have been reported (55,56). The chromatoplates are prepared in the usual manner (57) using silica gel G as the adsorbant. Development has been carried out in different solvent systems (Table II). The developed plates are dried and then observed under UV light (yellowish green). The plates are finally sprayed with a 0.5% Iodine-potassium iodide solution, the colors are observed in daylight (reddish brick-red) and under UV light, and the  $R_f$  values are determined (Table II).

Table II

Solvent system	R <sub>f</sub>
a- Toluene-ethylformate- formic acid ( 5 : 4 : 1 )	0.65
b- Benzene-ethylacetate ( 9 : 1 )	0.39
c- Benzene-acetone ( 9 : 1 )	0.71

A better resolution has been effected by two-dimensional chromatography on silica gel thin layers (Fig.6), and also by the use of wedge-shaped (56) chromatogram (Fig.7).

#### 7.73 Gas Liquid Chromatography

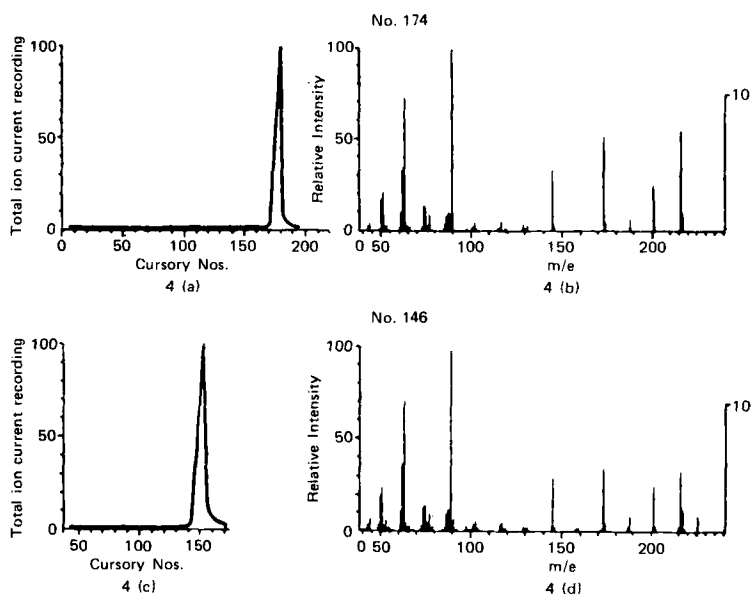
Stewart, and Shyluck (58) have developed a GLC method for the separation of methoxsalen and certain coumarins. The relative retention time of methoxsalen, relative to herniarin, is 3.6 under the following conditions: SEG column of copper tubing (0.61 m X 5mm o.d.) packed with succinate-ethyleneglycol polyester on a support of 60-80 mesh chromosorb W; column temperature, 208°; helium flow rate, 100 ml/min; injector temperature, 245°; and recorder sensitivity, 1 mv.

#### 7.74 High Performance Liquid Chromatography

A HPLC method has been reported (59) for the estimation of methoxsalen in the blood.

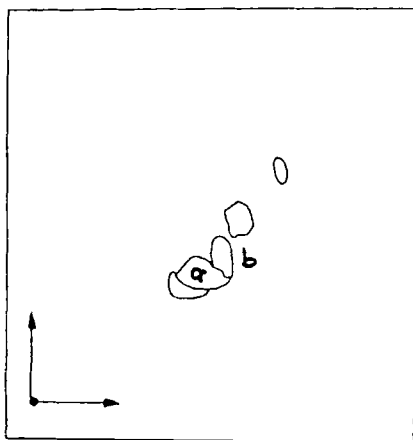
Another method for its determination in tablet dosage form has been also described (60). It

Fig. 5



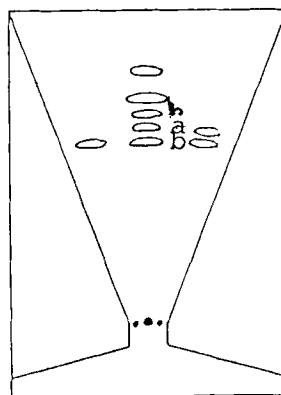
Gas Chromatogram and Mass Spectrometric fragmentation pattern of the drug recovered from plasma ( c and d ) and of an authentic Methoxsalen ( a and b ).

Fig. 6



Two-dimensional TLC showing a-Methoxsalen and b-Bergapten.

Fig. 7



Wedge-shaped TLC showing, a-Methoxsalen and b-Bergapten.

involves the use of a low volume positive displacement pump, universal injector, and a single wavelength detector (254 nm). A u Bondapak C<sub>18</sub> stainless steel column (3.8 mm x 30 cm) is used. The column temperature is ambient, the optical density is set at 0.05 a.u.f.s., the recorder is set at 10 mv full scale, and the chart speed is 0.25 cm per minute. The solvent (mobile phase) is methanol-water mixture (60:40), and the flow rate is controlled at 2 ml per minute. A typical chromatogram of methoxsalen, using Khellin as an internal standard, is shown in Fig.8. The recovery of methoxsalen has been found to be 86-95% of the stated amount.

#### 7.8 PMR Spectrometry

Loutfy and Hassan (11) have reported a rapid and simple PMR procedure for the estimation of methoxsalen in bulk drug and in pharmaceutical formulations. The method is based on the integration of the three methyl protons singlet of the 8-methoxy group of methoxsalen appearing at 4.35 ppm. Acetanilide, exhibiting three methyl protons singlet at 2.16 ppm, has been employed as an internal standard (Fig.9). Ethanol-free chloroform has been used as a solvent in the assay. The method is accurate, with a standard deviation of  $\pm 2.4$ , and an average recovery of 98.3%, in the tablet dosage form. The method proved to be reliable for the detection of the biologically inactive isomer, bergapten. This is attributed to the presence of 4-H proton doublet of bergapten appearing at 8.1 ppm. (Fig. 10)

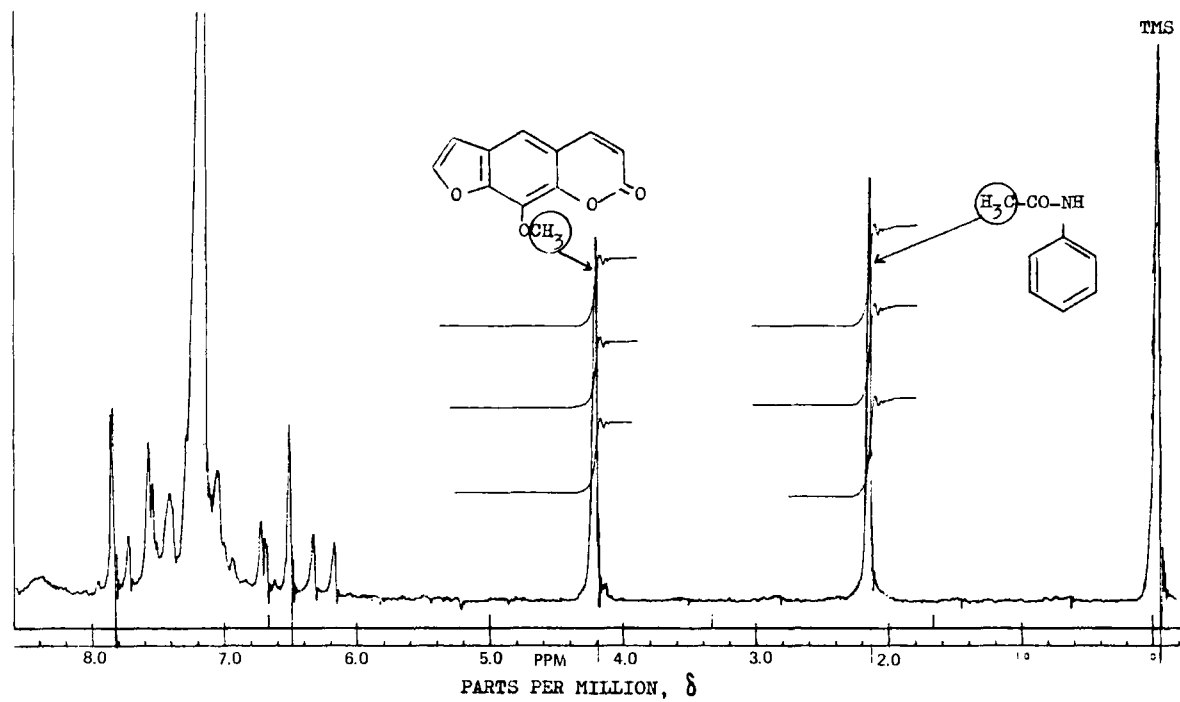


Fig. 9 - PMR Spectrum of Methoxsalen, Acetanilide and TMS in  $\text{CDCl}_3$ .

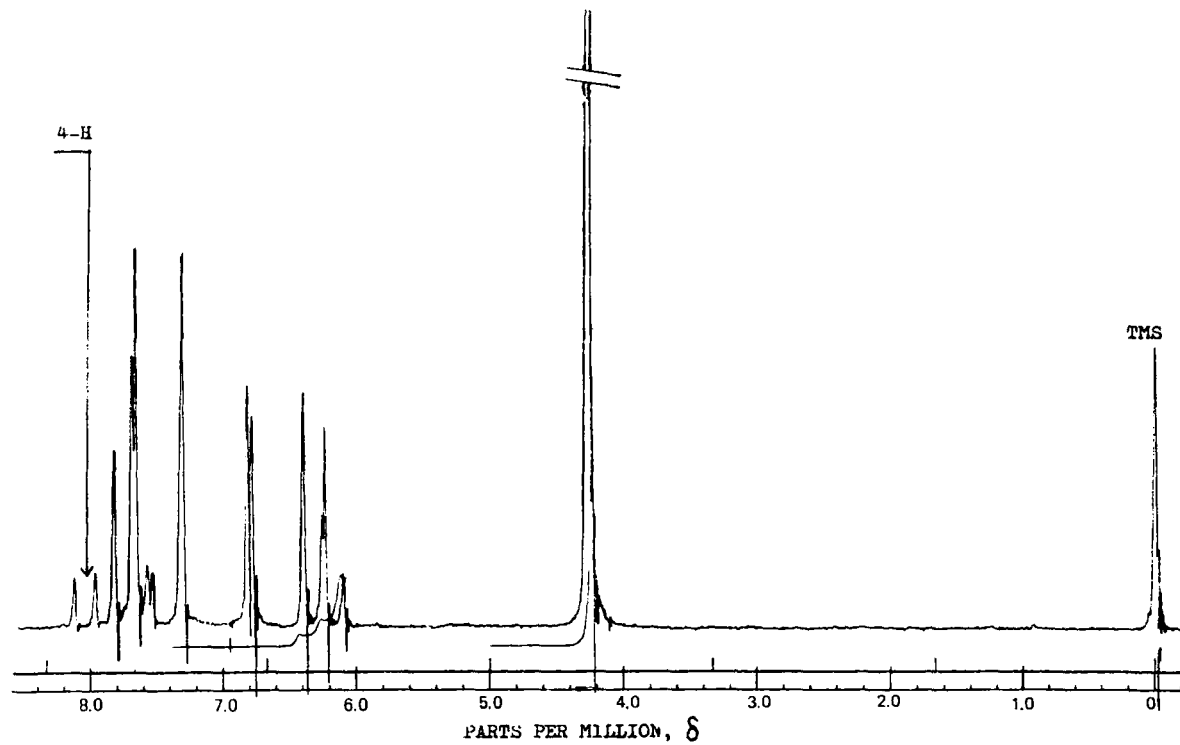


Fig. 10 - PMR Spectrum of Methoxsalen, Bergapten and TMS in  $\text{CDCl}_3$ .

This finding has contributed greatly to the specificity of the method.

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# NADOLOL

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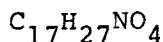
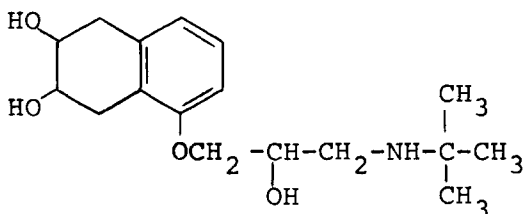
## 1. Introduction

### 1.1 History

Nadolol, a "β-blocking" antiarrhythmic agent, was synthesized,<sup>1</sup> tested<sup>2</sup> and developed in the laboratories of the Squibb Institute for Medical Research.

### 1.2 Name, Formula, Molecular Weight

Nadolol (SQ 11,725) is 2,3-cis-1,2,3,4-tetrahydro-5-(2-hydroxy-3-(tert-butylamino)propoxy)-2,3-naphthalenediol; CAS: 42200-33-9.



Molecular Weight 309.41

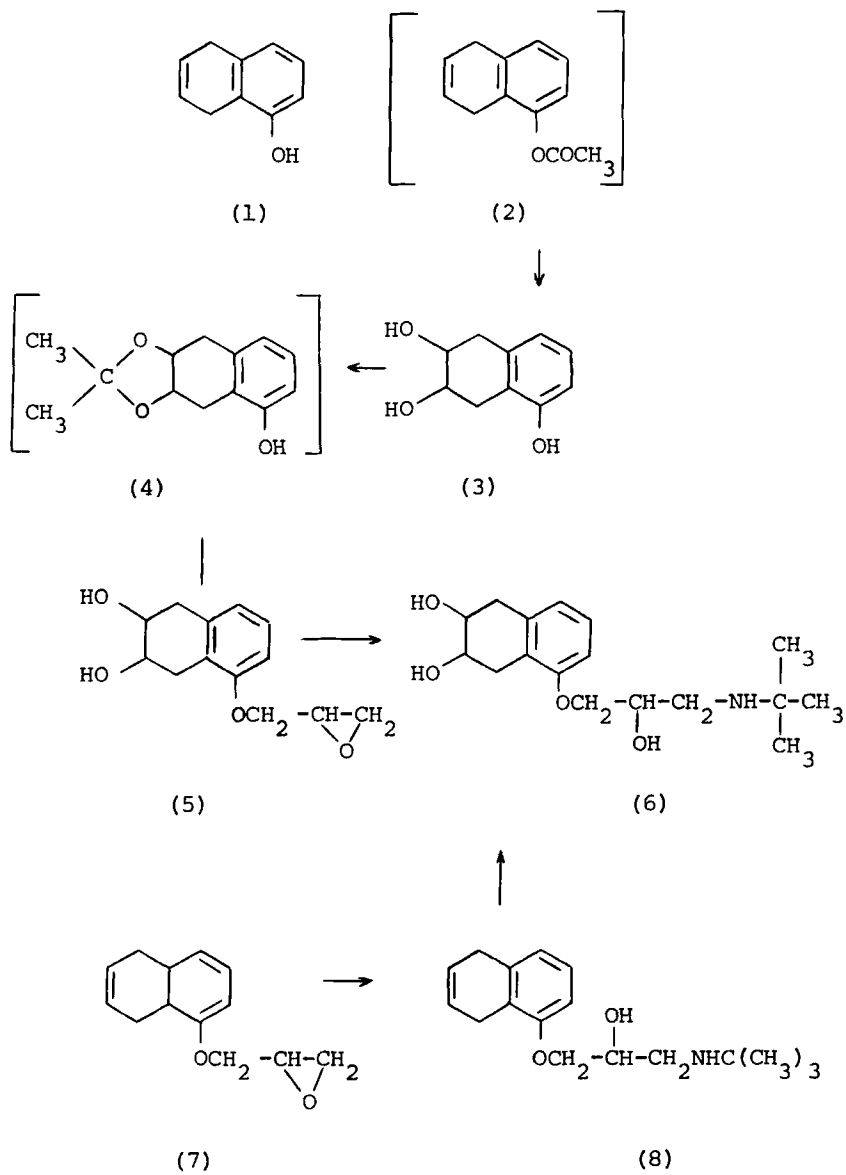
### 1.3 Appearance, Color, Odor

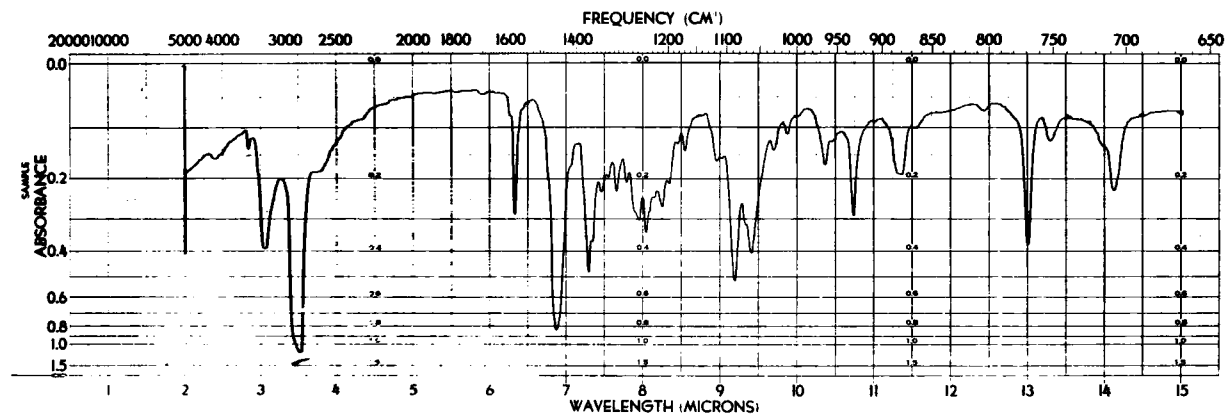
Nadolol is a white to off-white crystalline, odorless powder.

## 2. Synthesis

The multistep synthesis of nadolol is presented in Figure 1: 5,8-Dihydronaphthol (1) via its acetyl derivative (2) is converted to cis-5,6,7,8-tetrahydro-1,6,7-naphthalene triol (3). This in turn is converted with or without intermediate formation of the acetonide (4) to 2,3-cis, 1,2,3,4-tetrahydro-5-(2,3-epoxypropoxy)-2,3-naphthalene diol (5) which forms nadolol (6) on the addition of tertiary butylamine. For details, see reference 1. It can also be prepared by attaching t-butylamine to 5,8 dihydro-1-(2,3-epoxypropoxy) naphthalene (7) to form (8) and subsequent oxidation of the double bond and resolution to obtain nadolol (6).<sup>3,4</sup>

Figure 1  
Synthetic Pathways to Nadolol





Sq 11,725

Curve 70207

Mineral Oil Mull.

Figure 2. IR Spectrum of Nadolol, Mineral Oil Mull.  
Instrument: Perkin Elmer Model 21





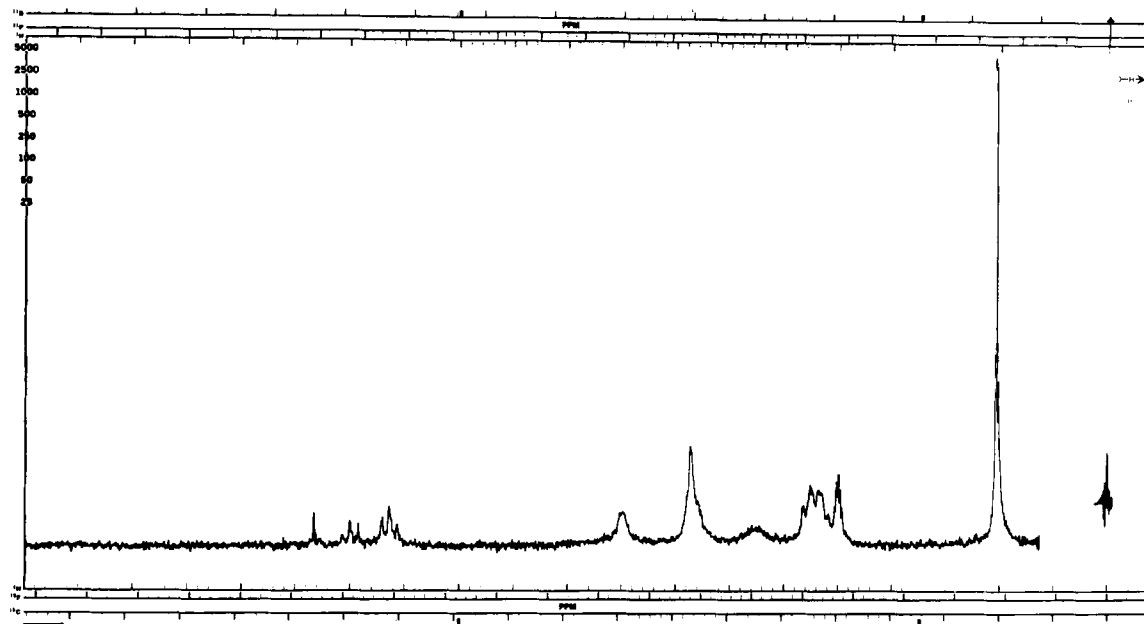


Figure 3. NMR Spectrum of Nadolol in DMSO- $d_6$ . Instrument: Varian XL-100-15  
Internal Standard: Tetramethylsilane

Table I (continued)

<u>Proton Position</u>	<u><math>\tau</math> Value*</u> (Coupling Constant, J, in Hz)
1	3.32d (8.0 Hz)
2	3.00t (8.0 Hz)
3	3.24d (8.0 Hz)
4 (3 protons)	5.53
5	6.75
6	7.28m
6'; 6''	7.28m
7	6.15b
7'	6.15b
8	8.97

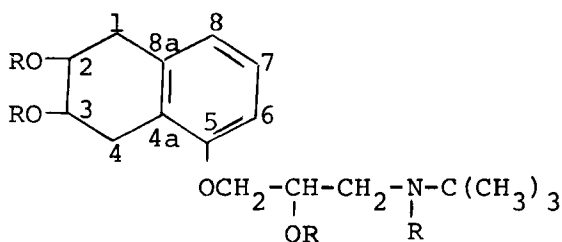
\*d-doublet; t-triplet; m-multiplet, b-broad

An inspection of the D<sub>2</sub>O exchange spectrum (Figure 4) shows disappearance of two resonances at  $\tau$ 5.53 and  $\tau$ 6.75. They correspond to the four interchangeable protons: three hydroxyls and one NH proton.

The C-13 NMR data of nadolol in dimethyl sulfoxide -d<sub>6</sub> (Figure 5) and its tetrabenzoate derivative in CDCl<sub>3</sub> (Figure 6) are compared in Table II.<sup>6</sup>

Table II

C-13 NMR Pattern of Nadolol and its  
Tetrabenzoate Derivative



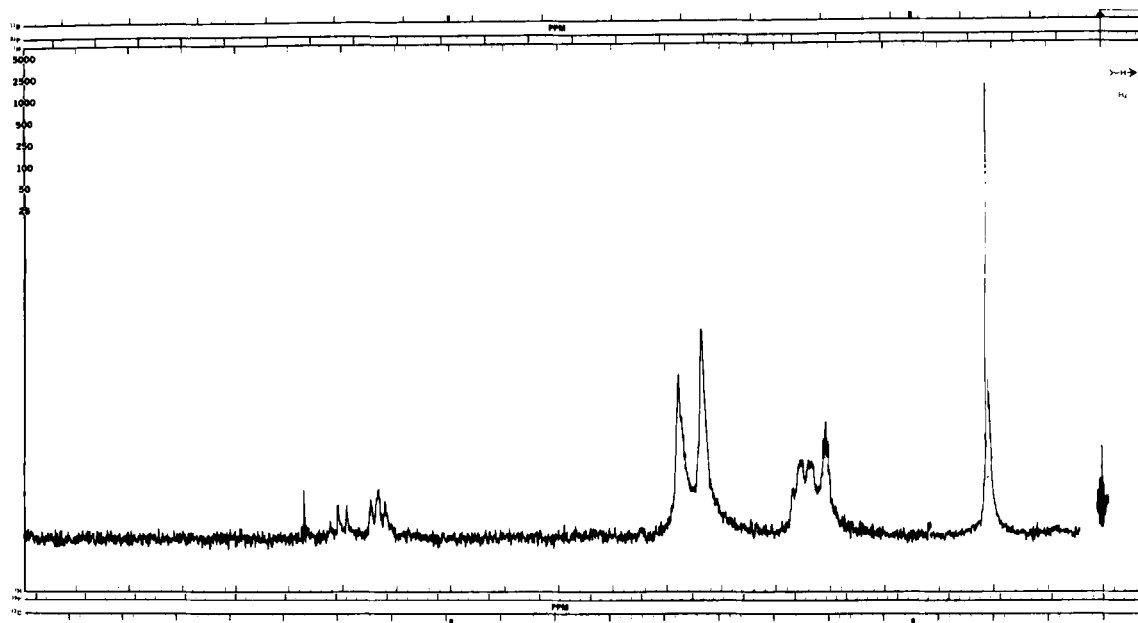


Figure 4. NMR Spectrum of Nadolol in DMSO-d<sub>6</sub>, D<sub>2</sub>O exchange.  
Instrument: Varian XL-100-15  
Internal Standard: Tetramethylsilane

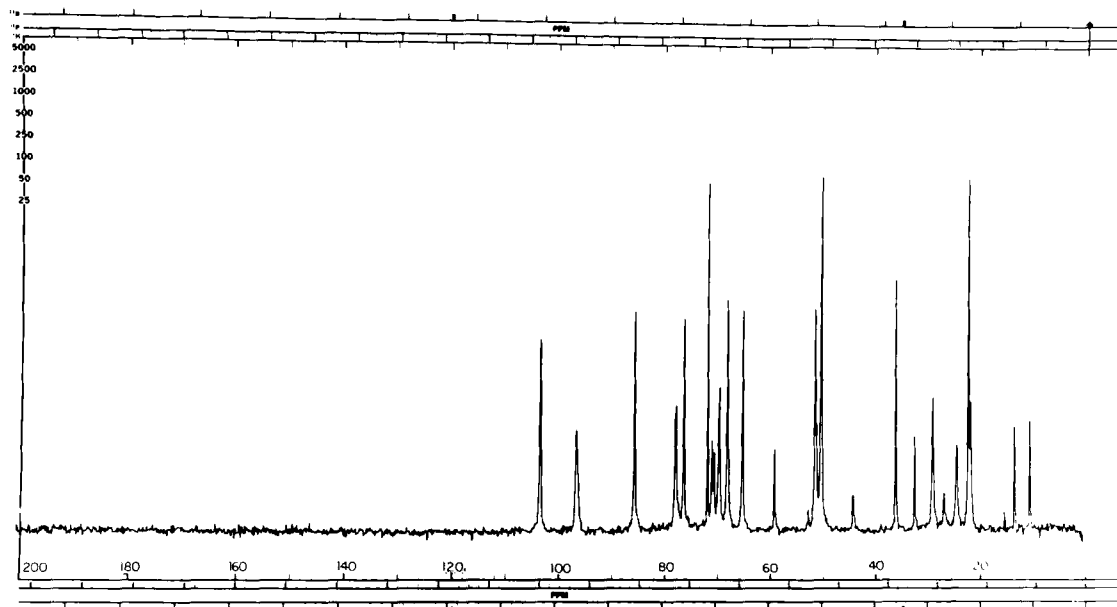


Figure 5. C-13 NMR Spectrum of Nadolol in DMSO-d<sub>6</sub>.  
Instrument: Varian XL-100-15, operated at 25.2 MHz

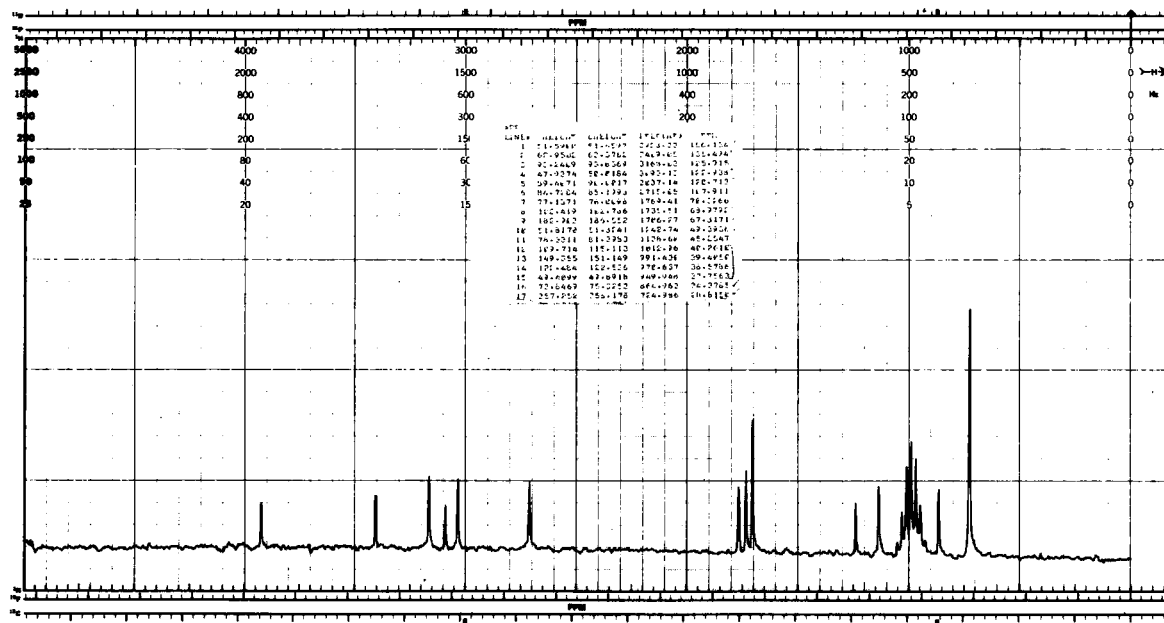


Figure 6. C-13 NMR Spectrum of Tetrabenzoate Derivative of Nadolol in  $\text{CDCl}_3$ . Instrument: Varian XL-100-15, operated at 25.2 MHz.

Table II (continued)

Carbon	Nadolol R=H	Tetrabenzoate R=COC <sub>6</sub> H <sub>5</sub>
Chemical Shifts, ppm.		
C-1	34.38	31.04, 31.27
C-2	67.82	69.86**
C-3	67.82	71.19**
C-4	28.81	26.60, 26.77
C-4a	122.94	121.12
C-5	156.13	155.37
C-6	107.91	107.91
C-7	125.92**	n.a.
C-8	120.71**	121.46
C-8a	135.49	138.70
OCH <sub>2</sub>	70.33	65.82, 66.23
CHO	68.98	69.10, 69.21
CH <sub>2</sub> N	45.25	47.47, 47.76
*C(CH <sub>3</sub> ) <sub>3</sub>	49.39	56.82
C(*CH <sub>3</sub> ) <sub>3</sub>	28.81	28.76

\* - indicates to which carbon the shift is ascribed.

\*\* - these carbons may be interchanged.

n.a. - not assigned.

The chemical shift assignments are made on the basis of non-decoupled spectra and known substituent effects. The data of the tetrabenzoate derivative show double resonances for side chain carbons (OCH<sub>2</sub>CH(OR)CH<sub>2</sub>N) as well as the tetrahydro carbons (>CH<sub>2</sub>). This can be ascribed to the presence of two racemates A and B in nadolol (see Sec. 3.3).

### 3.13 Mass Spectra

The low resolution mass spectrum of nadolol is shown on Figure 7. The high resolution mass spectrum yields a molecular ion at m/e 309.1956 with the formula C<sub>17</sub>H<sub>27</sub>NO<sub>4</sub> consistent with the assigned structure.

Typical of compounds containing t-butyl groups is the loss of 15 a.m.u. from the

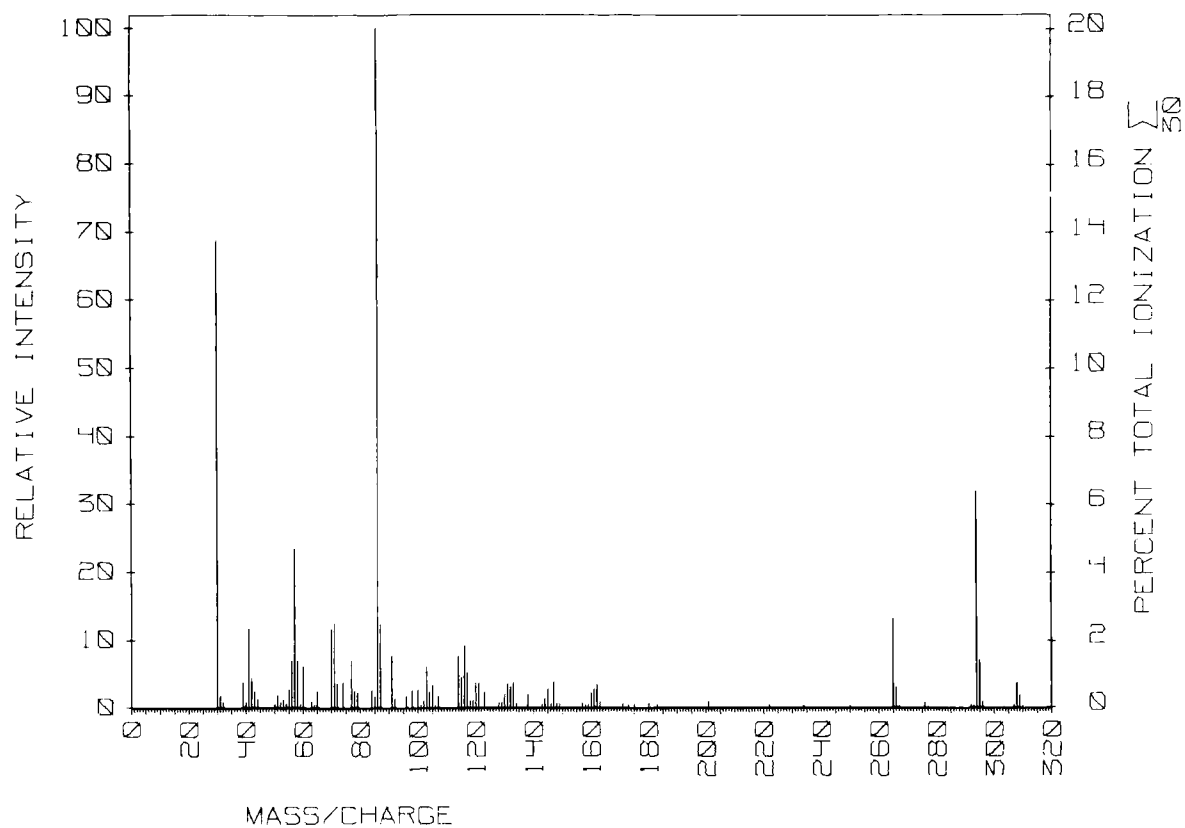
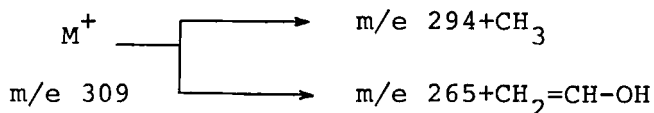
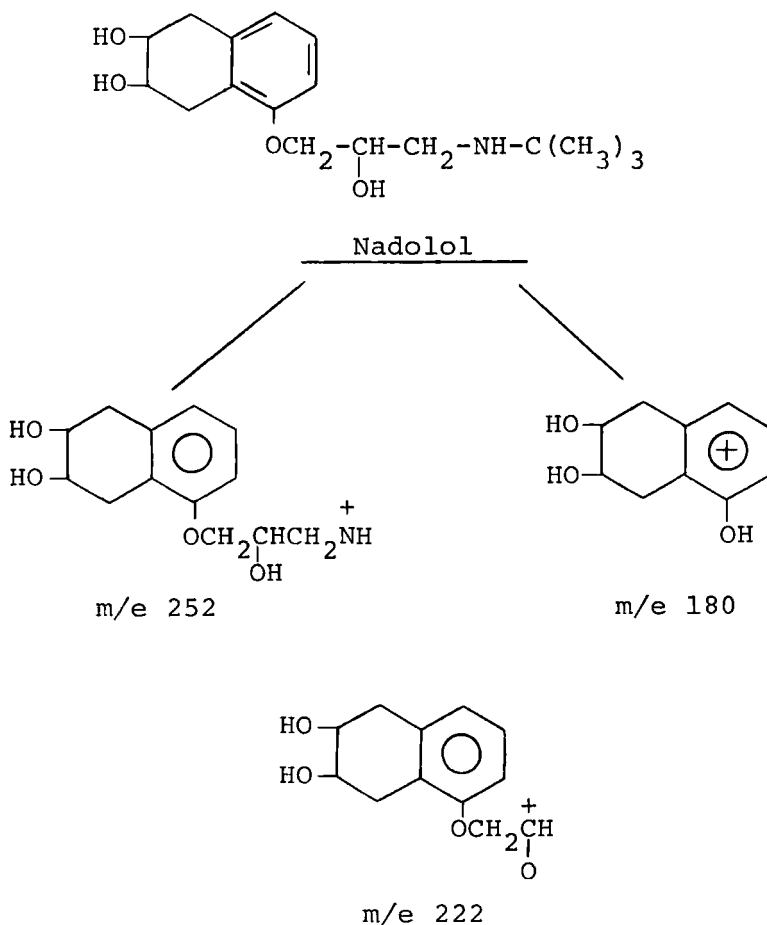


Figure 7. Low-Resolution Mass Spectrum of Nadolol.  
Instrument: AEI-MS-902

molecular ion to yield a peak at  $m/e$  294. The loss of 44 a.m.u. to give the peak at  $m/e$  265 (formula  $C_{15}H_{23}NO_3$ ) is interpreted as the elimination of  $CH_2 = CH - OH$  from the tetrahydronaphthalenediol portion of the molecule (see schematic below).

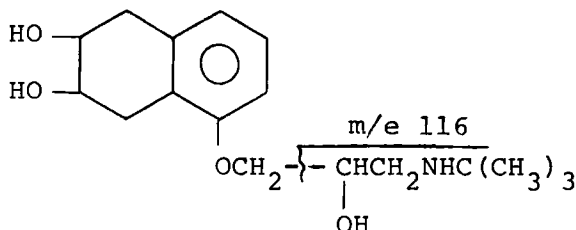


Fragmentation of the aliphatic side chain yields ions at  $m/e$  252, 222 and 180, the latter two accompanied by the proton transfer shown below:





A fragment ion at  $m/e$  116, corresponding to  $C_6H_{14}NO$ , can arise from the side chain cleavage depicted below:



### 3.14 Ultraviolet Spectrum

The ultraviolet spectrum of nadolol in methanol<sup>8</sup> is shown on Figure 8. It depicts a shoulder at 218 nm (concentration 0.017 mg/ml) and two well-defined peaks at 270 and 278 nm (concentration 0.171 mg/ml). The table below lists the absorbances in methanol:

$\lambda_{nm}$	$E_{1cm}^{1\%}$
219 (shoulder)	$\sim 275$
270	37.5
278	39.1

Inspection of this table reveals that the UV absorbance of nadolol is very low. It should also be noted that an appropriate blank correction would be necessary for the true  $E_{1cm}^{1\%}$  value at 219 nm (see also 4.31).

### 3.15 Fluorescence Spectroscopy

Nadolol has no native fluorescence in 95% ethanol, aqueous 0.1N sodium hydroxide or 0.1N hydrochloric acid. It can, however, be induced by heating samples at 100°C. in concentrated sulfuric acid (excitation maximum at 260 nm; emission maximum at 400 nm).<sup>8</sup> Nevertheless, this approach has not been utilized for analytical purposes because of interferences and variations in fluorescence.<sup>9</sup>

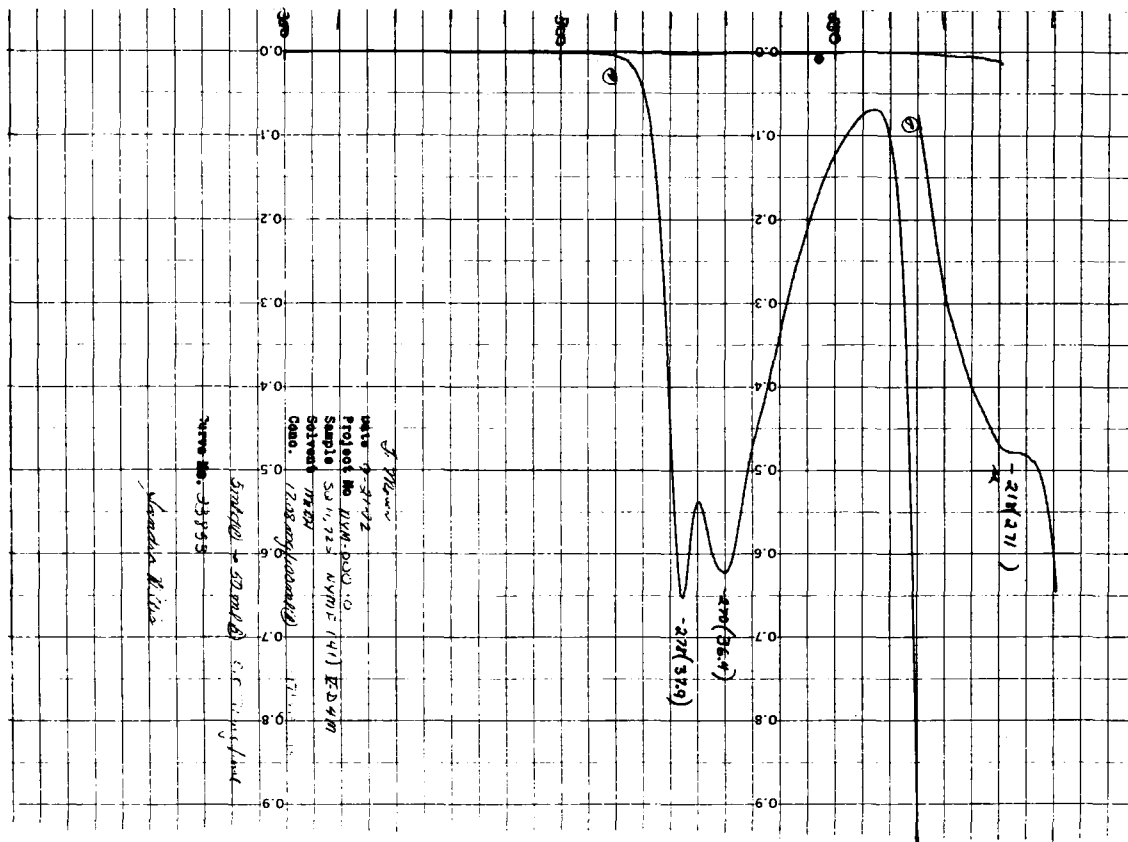


Figure 8. UV Spectrum of Nadolol in Methanol. Instrument: Cary 15

### 3.2 Solid State Properties

#### 3.21 Melting Range

Nadolol:	124-136°	C. <sup>1</sup>
Racemate A:	134-136°	C. <sup>10</sup>
Racemate B:	148-157°	C. <sup>10</sup>

#### 3.22 Differential Thermal Analysis (DTA)

Nadolol shows a single endotherm at about 130° C.<sup>11</sup> The two racemic compounds A and B showed endotherms at 140° C. and 146° C.,<sup>11</sup> respectively, which correlate with their melting ranges (see Section 3.21). The differential thermal analysis curves were recorded on a DuPont 900 Thermoanalyzer with a temperature rise of 15° C. per minute.

#### 3.23 Differential Scanning Calorimetry (DSC)

Attempts were made to determine the purity of nadolol by differential scanning calorimetry.<sup>11</sup> However, the results obtained were difficult to interpret due to the complex melting behavior exhibited by racemic mixtures.

#### 3.24 Polymorphism

No polymorphism has been reported for nadolol. However, it was observed<sup>12</sup> that an amorphous form of nadolol can be obtained by lyophilizing an aqueous solution of the compound. The amorphous nature of the lyophilate was ascertained through x-ray and thermal analysis. The amorphous form exhibited diffused melting behavior at 50° C. and was at least ten times as soluble in water at room temperature as the crystalline form.

#### 3.25 X-Ray Powder Diffraction

The x-ray powder diffraction patterns of nadolol (Table III, Figure 9) and racemic compounds A (Figure 10) and B (Figure 11) are presented.<sup>13</sup>

The diffraction patterns of the A and B racemates are quite different as can be seen from Figures 10 and 11. Consequently, an X-ray powder diffraction method was developed<sup>13</sup> to measure the percentages of racemate A and racemate B in samples of nadolol. The range of concentrations measurable

by this technique is 30% to 70% with an accuracy of  $\pm 5\%$  (see Section 3.3).

Table III

X-Ray Powder Diffraction Pattern of Nadolol\*

<u>2<math>\theta</math> (Deg.)</u>	<u>d (Å<sup>0</sup>)</u>	<u>I/I<sub>0</sub></u>
6.32	13.98	1.000
7.34	12.04	0.433
10.57	8.37	0.123
13.12	6.75	0.303
15.07	5.88	0.293
15.33	5.78	0.316
15.67	5.66	0.346
17.03	5.21	0.349
18.64	4.76	0.437
19.41	4.57	0.170
19.83	4.48	0.140
21.36	4.16	0.358
21.79	4.08	0.584
22.38	3.97	0.434
22.72	3.91	0.508
23.32	3.81	0.436
24.08	3.70	0.222
24.93	3.57	0.153
26.12	3.41	0.125
27.06	3.30	0.117
28.76	3.10	0.200
30.29	2.95	0.133
30.80	2.90	0.107
37.85	2.38	0.094

\*2 $\theta$  - Twice the angle of incidence or reflection.

d - Interplanar distance.

I/I<sub>0</sub> - Relative peak intensity based on highest intensity as 1.000

3.26 Single Crystal X-Ray Diffraction

Single crystal x-ray diffraction data of the hydrobromide salt of racemate A have been collected.<sup>14</sup> Crystals of the hydrobromide salt were large, well formed rods of the triclinic system. The unit cell dimensions were  $a = 7.822$  Å,  $b = 12.535$  Å,  $c = 9.712$  Å,  $\alpha = 101.8^\circ$ ,  $\beta = 93.1^\circ$ ,  $\delta = 89.2^\circ$ . There are two molecules in the unit cell, centrosymmetrically related.

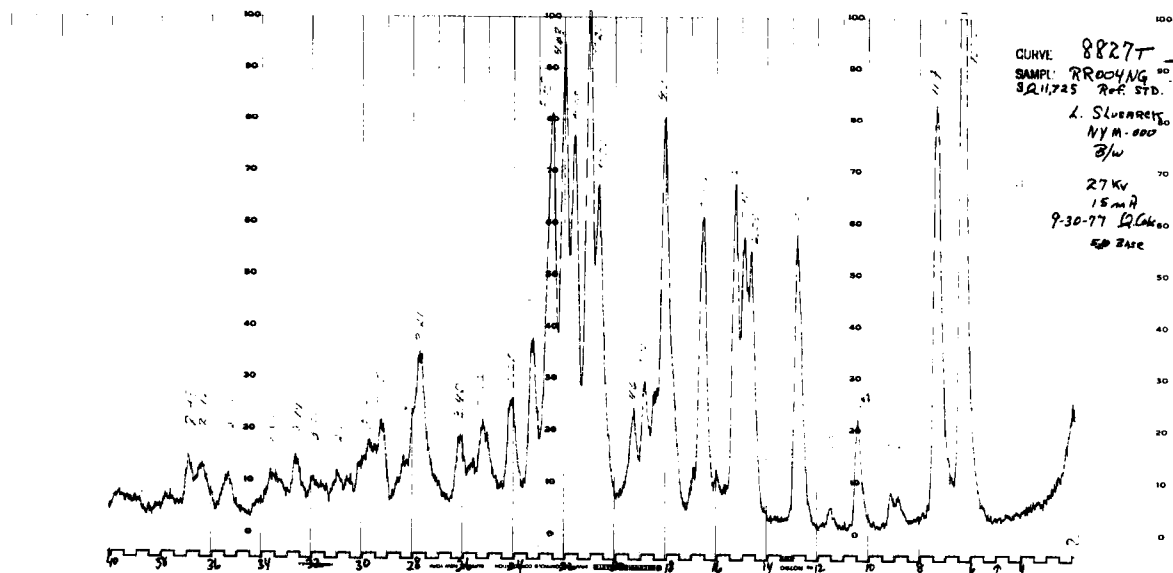


Figure 9. X-Ray Powder Diffraction Pattern of Nadolol.  
 Instrument: Phillips 120-101-11

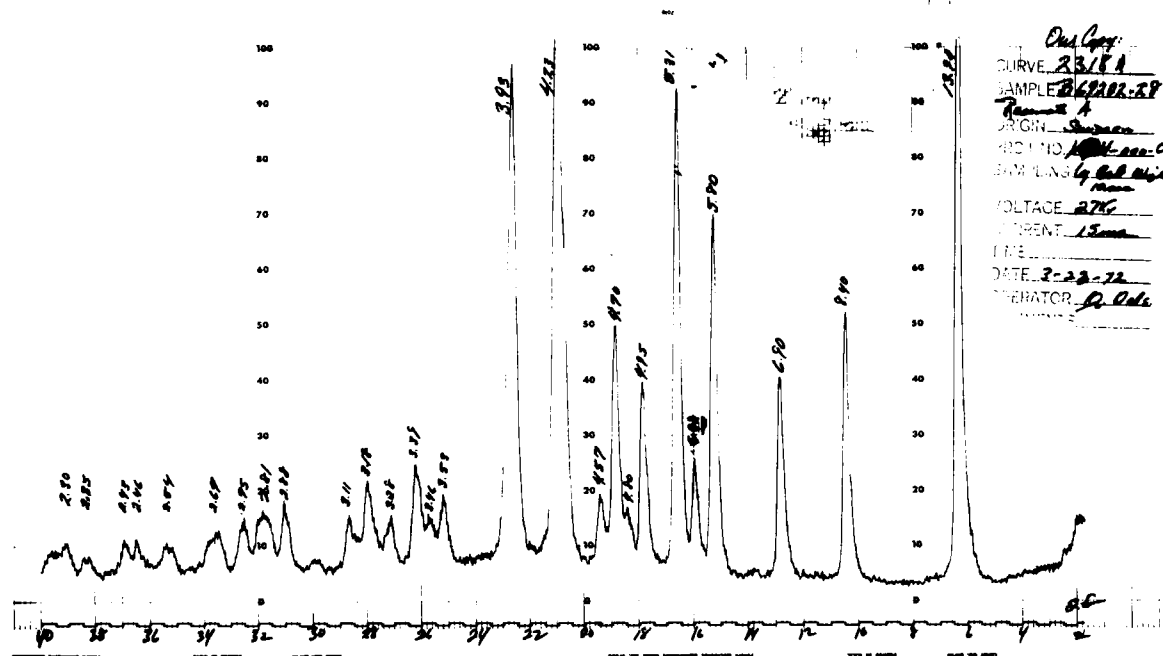


Figure 10. X-Ray Powder Diffraction Pattern of Racemate A of Nadolol.  
Instrument: Phillips 120-101-11

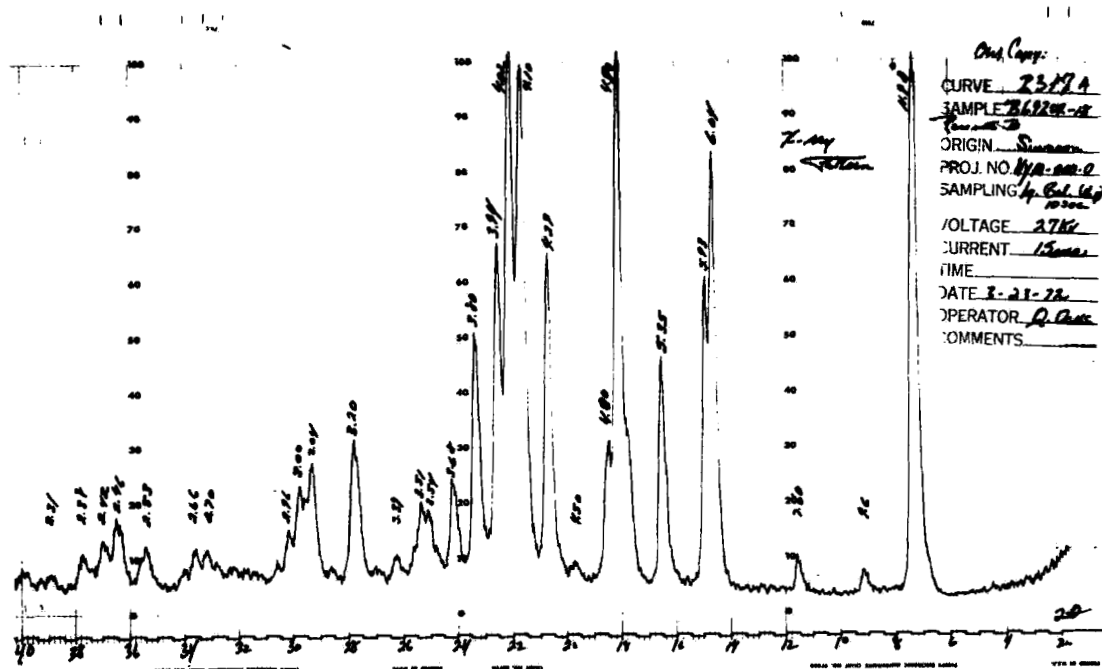


Figure 11. X-Ray Powder Diffraction Pattern of Racemate B of Nadolol.  
Instrument: Phillips 120-101-11

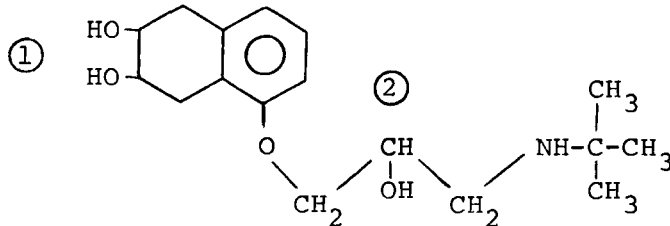
### 3.3 Racemate Composition

Nadolol consists of two sets of enantiomers. They are present as two racemic compounds: racemate A and racemate B.<sup>14</sup>

The following table illustrates the relative optical activity:

Table IV

#### Enantiomers of Nadolol

	
<u>Optical Rotation</u>	
Center ①	Center ②
+	+
-	-
+	-
-	+
	B
	A

The composition of the racemates can be determined by infrared spectroscopy of mineral oil mulls, powder x-ray diffraction (Section 3.25) or by NMR techniques. The infrared spectroscopic method<sup>5</sup> is based on the presence of specific absorption bands for racemate A and for racemate B:

A:  $1260\text{ cm}^{-1}$  ( $7.9\text{ }\mu$ )

B:  $1240\text{ cm}^{-1}$  ( $8.05\text{ }\mu$ ) and  $3580\text{ cm}^{-1}$  ( $2.8\text{ }\mu$ )

These bands are recognizable in mixtures of A and B in the range from 30%A - 70%B to 70%A - 30%B. Either one or both racemates may be measured independently with an accuracy of about  $\pm 5\%$ .

The NMR method of analysis is based upon the chemical shift difference of t-butyl groups of the tetrabenzoates of racemate A ( $\delta$  1.60) and racemate B ( $\delta$  1.57).<sup>6</sup> Quantitation of each racemate was obtained with  $\pm 2\%$  accuracy for samples contain-



ing 20 to 70% of racemate B. Structural assignments were made on the basis of europium shift reagent studies. Kiralshift reagent allowed for the separation of *t*-butyl group resonances of the *d,l* isomers of the side chains. Use of this reagent permits the determination of the optical purity of the side chain of racemate A and B or of the mixture of racemates.

An x-ray powder diffraction method for the quantitation of racemates was also developed<sup>13</sup> (see Section 3.25 and Figures 10 and 11).

An attempt was also made to separate racemates A and B by thin-layer chromatographic procedures.<sup>15</sup> Sufficient separation was not achieved, however, even after 202 solvent systems and 14 chromatographic adsorbents were examined.

### 3.4 Solution Data

#### 3.41 Solubility

Solubility data are summarized in Table V.<sup>12, 16, 17</sup>

Table V

#### Solubility of Nadolol

<u>Solvent</u>	<u>Temp. C°</u>	<u>Solubility mg/ml</u>
0.1N HCl	37	42.5
pH 5.0, 0.2M citrate	R.T.	40.1
pH 5.0, 0.2M phosphate	"	40.2
pH 7.0, 0.2M phosphate	"	30.4
Propylene glycol	37	97.5
50% Aq. PEG 400	"	46.0
Methylene Chloride	R.T.	2.0
Methanol	"	>200.0
Isopropanol	"	5.0
1,1,1-Trichloroethane	"	Insoluble
95% Ethanol	"	Freely soluble
Chloroform	"	Slightly soluble
Acetone	"	Insoluble
Benzene	"	Insoluble
Ethyl Ether	"	Insoluble
Hexane	"	Insoluble

### 3.42 pKa

A pKa value of 9.67 was determined potentiometrically.<sup>16</sup>

### 3.43 Partition Coefficient

The partition coefficient of nadolol was determined in the octanol/Krebs buffer system at room temperature.<sup>17</sup> The composition of Krebs buffer is the following: KCl-5mM; KH<sub>2</sub>PO<sub>4</sub>-1mM; NaHCO<sub>3</sub> - 26mM and NaCl-122mM. The table below shows the results obtained:

<u>Partition Coefficient</u>	<u>pH</u>
0.25	8.1
1.3	8.7

## 4. Analytical Tests and Methods

### 4.1 Elemental Analysis

The following results were obtained on a Squibb Research Standard:<sup>10</sup>

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	66.99	65.92
H	8.80	8.76
N	4.53	4.38

### 4.2 Identification Tests

Identification of nadolol in tablet formulations is based on a color reaction of the oxidized drug with phenylhydrazine and ferricyanide.<sup>18</sup> The cis-hydroxy groups are first oxidized to aldehydes with periodate and then reacted with phenylhydrazine to form a hydrazone. In acid solution, the hydrazone gives a red color with potassium ferricyanide.

Thin-layer chromatography<sup>19</sup> (Section 4.61) and infrared spectroscopy (Section 3.11) have also been used to identify the drug.

### 4.3 Spectrophotometric Analysis

#### 4.31 Ultraviolet Analysis

Nadolol displays three absorption peaks in the ultraviolet region at about 218, 270 and 278 nm (Section 3.14). Although the molar absorptivity of nadolol is quite low, it is adequate

for the study of dissolution rates of nadolol tablets.<sup>20</sup> Beer's law is obeyed up to at least 4 mg of nadolol/100 ml, as measured in pH 1.2 hydrochloric acid at 277 nm.<sup>21</sup>

#### 4.32 Colorimetric Methods

Complexation of the amino group of nadolol with bromophenol blue in chloroform yields a yellow color with an absorption maximum at 414 nm. This is of potential usefulness for a quantitative assay of nadolol in formulation.<sup>18</sup>

A colorimetric assay for the determination of nadolol in tablet formulation is based on a hydrazone absorption at 352 nm in chloroform.<sup>22</sup> The two vicinal hydroxyl groups are oxidized to the corresponding dialdehyde, which is condensed with 2,4-dinitrophenylhydrazine yielding the hydrazone.

#### 4.33 Fluorescence Spectrophotometric Analysis

Although nadolol does not exhibit native fluorescence, it can be modified to yield a strongly fluorescent derivative. A fluorometric assay for the quantitation of nadolol in serum and urine at nanogram and microgram levels has been described.<sup>23</sup>

The drug is oxidized with periodic acid to the corresponding dialdehyde and coupled with o-phenylenediamine to produce a fluorescent compound. Using a suitable filter, the emission peaks of the reagents and nadolol derivative are well separated ( $\lambda$  excitation = 305 nm and  $\lambda$  emission = 445 nm).

#### 4.4 Titrimetric Methods

##### 4.41 Reaction with Chloramine-T<sup>8</sup>

Nadolol is oxidized with Chloramine-T and the excess reagent is reacted with potassium iodide. The liberated iodine is titrated with sodium thiosulfate. The mechanism of the reaction of the drug with Chloramine-T is not known. This reaction can be used for the determination of nadolol in tablet formulations. However, the more readily controlled colorimetric method is preferable (Section 4.32).

#### 4.42 Nonaqueous Titrations

By virtue of the presence of an amino group, titration with acetic perchloric acid can serve to quantitate nadolol.<sup>24</sup> Quinaldine red or crystal violet indicators are used to determine the end-point. The amino group is titrated indirectly.<sup>25</sup> First, an ammonium salt of nadolol is formed with glacial acetic acid. Then, the released acetate ion is titrated with perchloric acid to the end-point monitored potentiometrically or with an internal indicator. The method has good precision and the results obtained using both indicators were comparable. It was used to develop bulk, batching and formulation assays.

#### 4.5 Gas Chromatography/Mass Spectrometry

A method to determine the serum concentration of nadolol by selected ion monitoring (SIM) and gas chromatography/mass spectrometry (GC/MS) of the tri(trimethylsilyl) ether derivative has been described.<sup>26</sup> The drug is extracted from serum and a known amount of internal reference, N-methyl-nadolol, is added. After lyophilization of the acidic extract, the resulting solid is reacted with N-trimethylsilylimidazole. The m/e 86 fragment ion of nadolol and the m/e 100 ion of the internal reference N-methyl-nadolol are monitored to establish the relative concentration ratio.

The detection level of this method is 2.6 ng/ml. No interferences are detected from extracts of fresh human serum at the relatively low mass ions of m/e 86 and 100. However, significant interferences were observed with several commercial serum samples at these masses. They probably result from contamination by plastic or rubber components used during the serum processing. Parallel measurements by spectrofluorometry<sup>23</sup> (Section 4.33) on duplicate samples, demonstrate a correlation coefficient of 0.9.

#### 4.6 Chromatographic Methods

##### 4.61 Thin-Layer Chromatography

A thin-layer chromatographic method has been developed<sup>15</sup> to measure quantitatively the purity of nadolol samples. The TLC separation is achieved on silica gel GF plates using the solvent system acetone-chloroform-2N ammonium hydroxide

(80:10:10). The position of the nadolol zone is located under short-wave ultraviolet light (maximum at  $\sim 254$  nm). The isolated zone is eluted with 95% ethanol and the absorbance of the eluate is measured at 278 nm. This procedure provides an excellent separation of ultraviolet absorbing impurities and allows for the quantitative measurement of the drug. This assay has been adapted for measuring the stability of nadolol in tablet formulations.

As mentioned in Section 3.3, attempts to separate the two racemates of nadolol by TLC were unsuccessful.<sup>15</sup>

#### 4.62 Gas Chromatography

A gas chromatographic method has been developed<sup>27</sup> for the quantitative measurement of nadolol in solutions. The drug is extracted with dichloromethane, filtered and evaporated together with added brompheniramine maleate as an internal standard. After evaporation to dryness, the trimethylsilyl derivative is formed. The GC parameters are as follows: oven temperature is  $210^{\circ}$  C. and the circular glass column is 1.7 m with 3 mm i.d., packed with 3% (w/w) OV-17 on 60-80 mesh Gas Chrom Q (silanized). Retention times of a typical run are: nadolol-8.5 min and brompheniramine standard - 4.5 min.

#### 4.63 High Pressure Liquid Chromatography

An HPLC method for the quantitative determination of nadolol has been developed.<sup>28</sup> A reverse phase ethylsilane column was used, operated at pressures of 200 to 2,000 psi and equipped with a precision loop injector and a fixed wavelength (254 nm) or variable wavelength (220 nm) detector. As mobile phase, a 35% methanol-65% aqueous 0.0005M hydrochloric acid-0.05M sodium chloride solution was used.

### 5. Stability - Degradation

#### 5.1 Solid State Stability

Nadolol exhibits excellent stability as a solid.<sup>29</sup> There was no apparent degradation of the bulk samples which were held at high temperatures for prolonged periods. The same TLC pattern was obtained for samples held at  $5^{\circ}$  C. and at  $50^{\circ}$  C. for

over two years.<sup>10</sup> Results of a light stability study<sup>12</sup> shows that nadolol and its racemic composition are stable under 900 foot candle light. Visual examination of a sample exposed to light for 6 months showed slight discoloration.

## 5.2 Solution Stability

Lyophilized sterile solutions of nadolol in 0.1M, pH 7.4 sodium phosphate buffer, showed no evidence of decomposition when held at room temperature for 51 days.<sup>12</sup> In unbuffered solutions, samples prepared at various pH's were stable after 3 months' storage at 50° C.<sup>30</sup> A very slight discoloration was noted in some samples after 3 months at 50° C. Storage of nadolol solutions at 80° C. for 2 months produces degradation and discoloration at most pH's. Exposure to intense light results in discoloration of solutions at pH 2, 2.92 and 9.8, after 2 weeks' storage. Variation in the pH values with temperature and time are below 1 pH unit for most solutions with the exception of those stored at 80° C.

## 6. Analysis of Body Fluids

A sensitive fluorometric method, capable of measuring microgram or nanogram levels of nadolol in human urine and serum has been developed<sup>23</sup> (Section 4.33). There is no interference in this assay from: dialyzing medium used during the clinical study, the diuretics hydrochlorothiazide and furosemide, and epinephrine and norepinephrine.<sup>3</sup> This fluorometric method has been adapted for nadolol determinations in human bile at levels from 0.005 to 5 µg/ml.<sup>8</sup>

Another technique, Selected Ion Monitoring Gas Chromatography/Mass Spectrometry, is described in Section 4.5, for application to nadolol quantitation in serum.<sup>26</sup> Suitable detection levels are obtained and no interferences from blood components or other administered drugs are observed. The SIM-GC/MS method shows lower detection limit and better sensitivity than the spectrofluorometric assay. Both SIM-GC/MS and fluorometric methods, in the absence of fluorescing metabolites, yield equivalent results. The fluorometric method is more adaptable to processing a large number of samples while the SIM-GC/MS method should be selected where specifi-

city is required or where the serum levels are extremely low.

#### 7. Drug Metabolism

Metabolic studies<sup>31</sup> with nadolol-<sup>14</sup>C were carried out in patients at a dose that could safely be given both orally and intravenously. Maximum concentrations of radioactivity were attained in plasma 2 to 4 hours after drug administration. When given intravenously, concentrations of radioactivity decreased rapidly during the first hour after drug administration, reflecting distribution of radioactivity into tissues. Terminal plasma half-times are an average 12.2 hours after oral and 9.8 hours after intravenous administration. After oral doses, an average of 24.6% and 76.9% of the dose is excreted in urine and feces, respectively, whereas, after intravenous doses, an average of 72.9% and 23.3% of the dose was excreted by the same route.

The radiolabeled drug is excreted unchanged in the urine and feces after either oral or intravenous administration indicating no biotransformation of the drug.

The metabolism of nadolol has also been studied in rats, dogs and monkeys.<sup>32, 33</sup>

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# NITRAZEPAM

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## 1. Description

### 1.1. Nomenclature

#### 1.11 Chemical Names

1,2-Dihydro-7-nitro-2-oxo-5-phenyl-3H-1,4-benzodiazepine.

1,3-Dihydro-7-nitro-5-phenyl-2H-1, 4-benzodiazepin-2-one.

#### 1.12 Generic Name

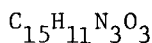
Nitrazepam

#### 1.13 Trade Names

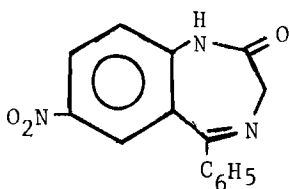
Benzalin, Calsmin, Eunoctin, Megadon, Mogadon, Mogadan, Nelbon, Nitrenpax, Paxisyn, Pelson, Radedorm, Relact, Sonebon, Sonnolin.

### 1.2 Formulae

#### 1.21 Empirical



#### 1.22 Structural



### 1.3 Molecular weight

281.26

### 1.4 Elemental Composition

C, 64.05%; H, 3.94%; N, 14.94%; O, 17.07%

### 1.5 Appearance, color, odor

A yellow, crystalline powder, odorless.

## 2. Physical properties

### 2.1 Crystal properties

#### 2.11 Crystallinity

Parch and Lapysh (1) had described micro-crystallographic reaction, for the detection of nitrazepam (detection limit 0.1 ug) and other benzodiazepine derivatives. This is based on evaporating a solution of the sample, on a watch-glass, the residue is kept for 5 to 10 minutes after adding one drop of 0.1 N-HCl, then one drop of a suitable reagent solution is added and the mixture is set aside in a moist atmosphere. The various types of crystals formed have been described.

#### 2.12 Melting Point

224-226°C (2); 226-229°C (3)

### 2.2 Solubility

Nitrazepam is soluble in alcohol, acetone, chloroform, and ethyl acetate; insoluble in water, ether, benzene, and hexane (3,4).

### 2.3 Identification

B.P. 1973 (3) specifies the following identification tests for nitrazepam:

- a) The infrared absorption spectrum exhibits maxima which are only at the same wavelengths as, and have similar relative intensities to, those in the spectrum of nitrazepam authentic specimen.
- b) The light absorption, in the range 230 to 250 nm, of a 2-cm layer of a 0.0005% w/v solution, in a mixture of 1 volume of N hydrochloric acid and 9 volumes of methyl alcohol, exhibits a maximum only at 280 nm; extinction at 280 nm, about 0.91.

- c) To 10 mg add 5 ml of hydrochloric acid and 10 ml of water, heat on a water-bath for 15 minutes, and filter. To the clear filtrate add 1 ml of a 0.1% w/v solution of sodium nitrite, allow to stand for 3 minutes and add 1 ml of a 0.5% w/v solution of sulfamic acid. Allow to cool for 3 minutes and add 0.1% w/v solution of N-(1-naphthyl) ethylenediamine hydrochloride, a red colour is produced.

## 2.4 Spectral properties

### 2.41 Ultraviolet Spectrum:

Nitrazepam, in neutral methanol solution, shows maxima at 218, 258 nm, and an inflection at about 308 nm (Fig. 1).

Nitrazepam, in ethanol, exhibits (4) maxima at 218, 260 nm; minimum at about 242 nm. In 0.1N sulphuric acid, the drug shows a maximum at 277.5 nm  $E_{1\%}^{1cm} = 1500$  and an inflection at about 340 nm.

The UV absorption spectrum of nitrazepam is used as a mean of identification and assay of the drug in tablet formulation in B.P. 1973 (3).

### 2.42 Infrared spectrum

The IR spectrum of nitrazepam is shown in Fig. 2. The spectrum was obtained from Nujol mull. The structural assignments have been correlated with the following band frequencies:

Frequency ( $\text{cm}^{-1}$ )	Assignment
1680	C=O
1600	C=C aromatic
1370	NO <sub>2</sub>

Clarke (4) has cited the following characteristic finger-print bands for nitrazepam when determined in potassium bromide disc:

1352, 1692, 702, and 1615  $\text{cm}^{-1}$

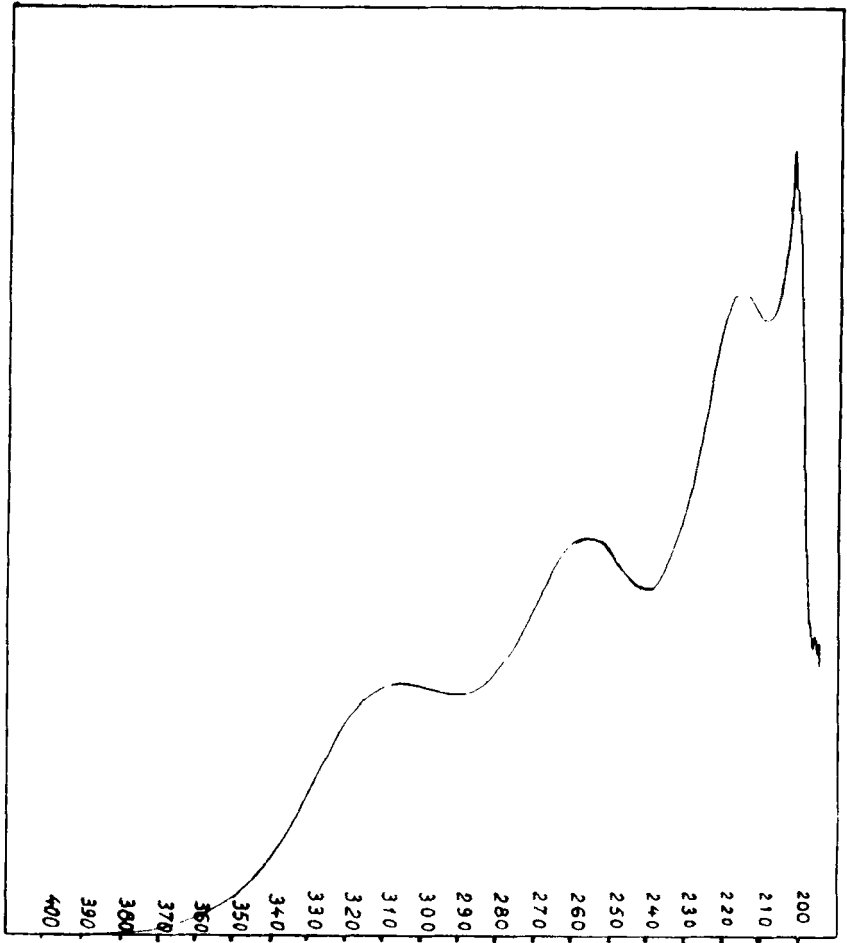


Fig. 1 - Ultraviolet spectrum of Nitrazepam in methanol

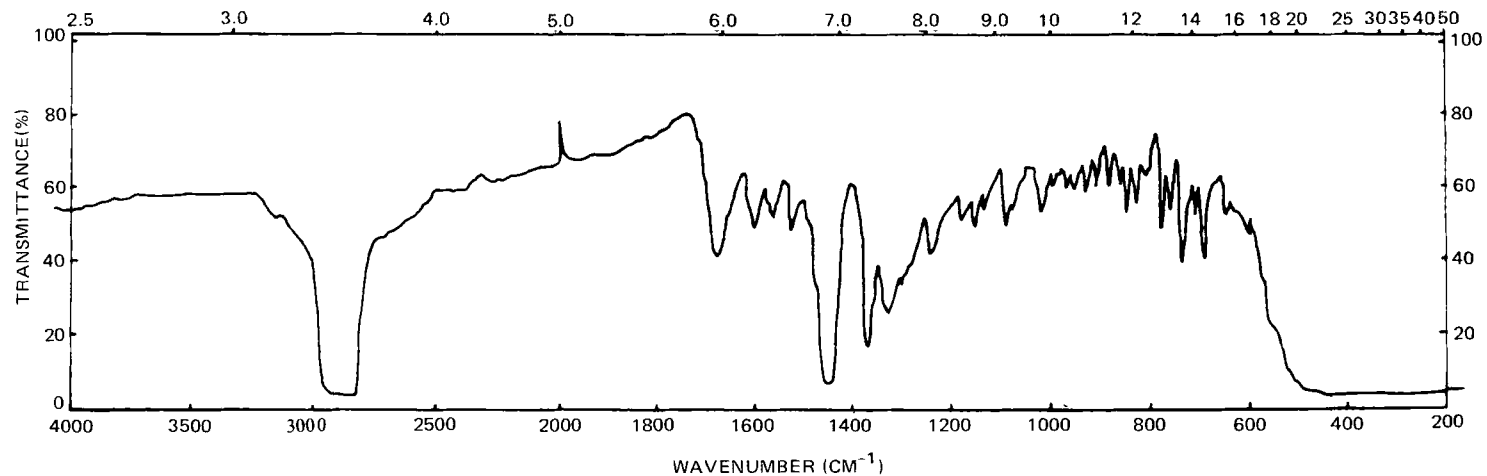


Fig. 2 - Infrared spectrum of Nitrazepam in nujol mull.



2.43 Nuclear Magnetic Resonance Spectrum

A typical NMR spectrum of nitrazepam is shown in Fig.3. The sample was dissolved in deuterated chloroform ( $\text{CDCl}_3$ ). The spectrum was determined on a Varian T-60A NMR spectrometer with TMS as the reference standard.

The following structural assignments have been made for Fig.3:

Chemical Shift ( $\delta$ )	Assignment
4.4 (singlet)	$\text{CH}_2$ at $\text{C}_3$
7.2 (singlet)	C-H aromatic at $\text{C}_9$
7.4 (doublet)	Five aromatic protons of the phenyl group at $\text{C}_5$ .
8.2 (singlet)	Two aromatic protons at $\text{C}_6$ and $\text{C}_8$
10.1 (broad singlet)	N - H

2.44 Mass spectrum and fragmentometry

The low resolution mass spectrum of nitrazepam is shown in Fig. 4. It was obtained on a Finnigan 1015 L quadrupole mass spectrometer of an ionisation potential of 70 eV. The spectrum shown was obtained by direct insertion of nitrazepam. It shows a molecular ion  $\text{M}^+$  at  $m/e$  281 (relative intensity 42.8%) and  $\text{M}^+ + 1$  at  $m/e$  282 (relative intensity 8.1%). Some of the most prominent ions are given in Table I.

Table I

<u><math>m/e</math></u>	<u>Fragment</u>
280	M-H
264	M-OH
254	M-HCN
253	M-(H,HCN)
252	M-(H,CO)
235	M- $\text{NO}_2$

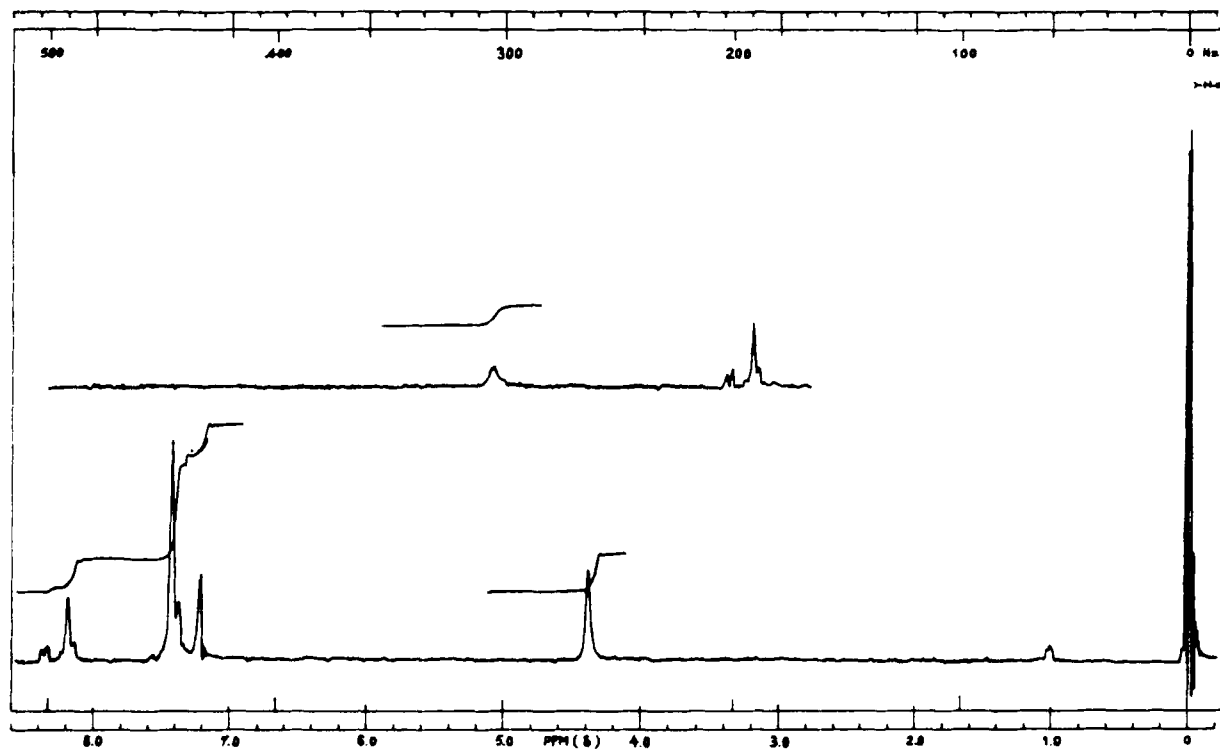


Fig. 3 - NMR spectrum of Nitrazepam in CDCl<sub>3</sub> containing TMS as an internal standard.

12334 DA SCAN 35 SIGMA=8 RT=2.7 BACKGD=145X100% 100%= 66560  
TITLE: NITRAZEPAM M.W. 281.26

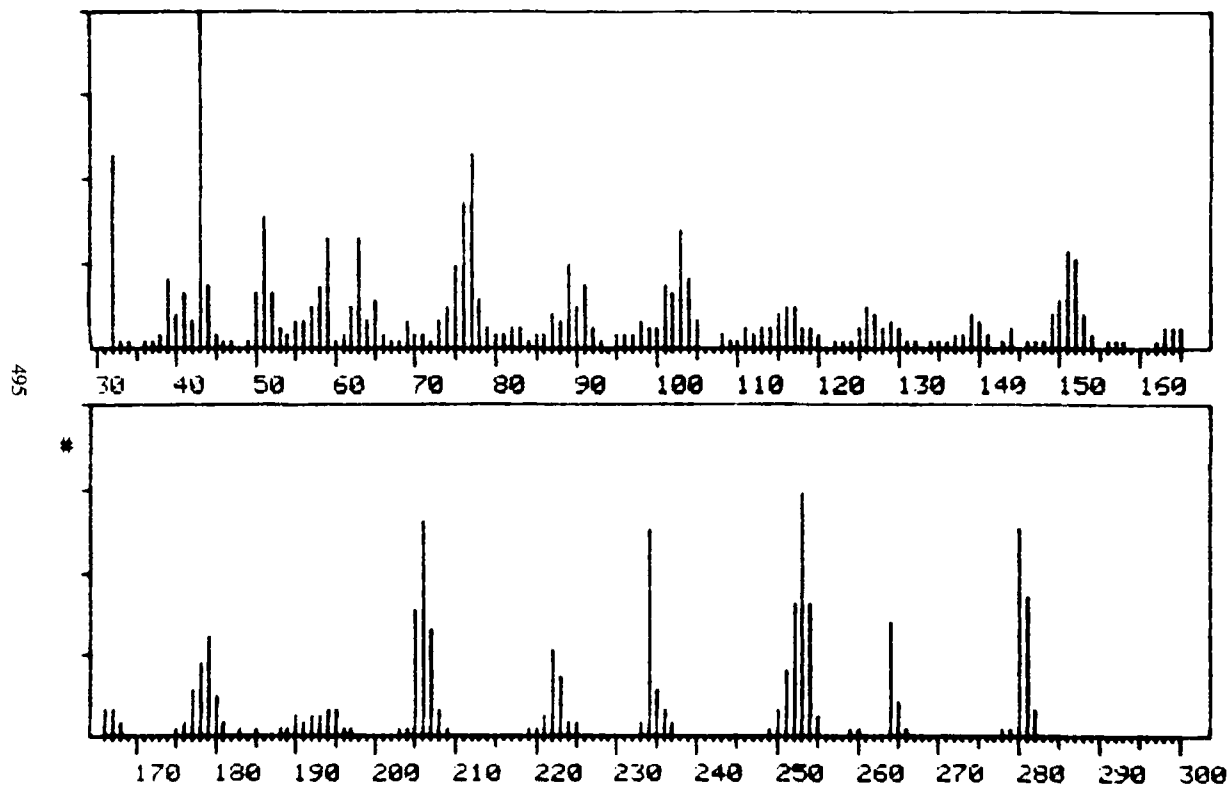
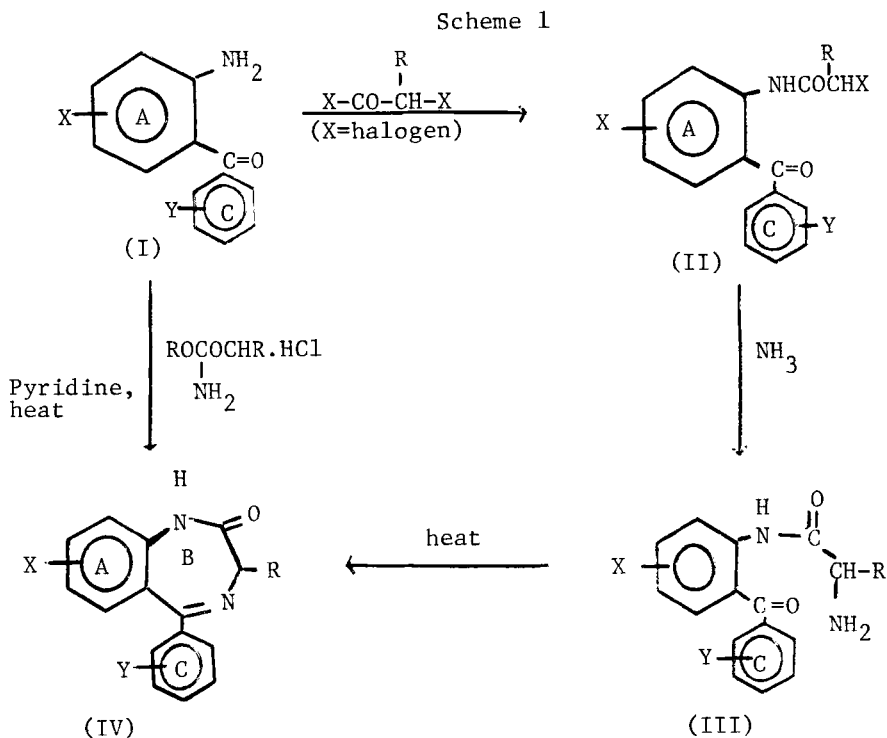


Fig. 4 - Mass spectrum of Nitrazepam (EI) - direct probe.

<u>m/e</u>	<u>Fragment</u>
234	M-(H,NO <sub>2</sub> )
207	M-(NO <sub>2</sub> -CO)
206	M-(H-NO <sub>2</sub> -CO)

### 3. Synthesis

The two most frequently used methods, with good yields (5,6), for the synthesis of simple benzodiazepinones are shown in Scheme 1.



As can be seen, in both cases, 2-aminobenzophenones are used as starting materials. Treatment of the appropriately substituted aminobenzophenone with a haloacetyl halide yields a compound II which, on treatment with ammonia, gives the benzodiazepinone IV via an amino derivative III. This method generally gives better overall yields of up to 70-80%, although it involves more steps. Another extensively used method is the treatment of 2-aminobenzophenone with an amino acid ester hydrochloride in pyridine, lead-

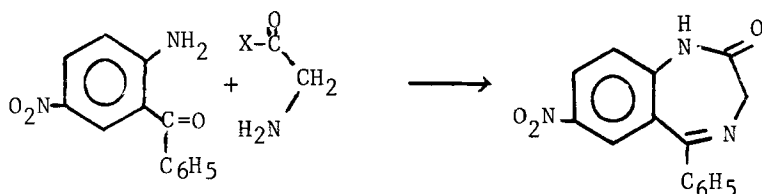
ing directly from I to IV.

Other routes for the construction of the 7-membered ring, which have been developed subsequently, involve the use of intermediates possessing a protected or potential glycine moiety (7,8).

Nitrazepam has been prepared by the following method (9):

Anhydrous hydrochloric acid is put into a stirred mixture containing 2-amino-5-nitrobenzophenone, glycine, and pyridine. The reaction mixture is refluxed for more than 48 hours, at intervals, and then concentrated under vacuum. The residue is partitioned between benzene and water. The benzene layer is washed with water and dried over anhydrous magnesium sulphate and then concentrated under vacuum to give the dried product.

Nitrazepam is also prepared by the treatment of 2-amino-5-nitrobenzophenone with a  $\beta$ -acylaminoethyl halide (10).



#### 4. Stability and Decomposition Products:

Beyer and Sadee (11) have published a monograph giving the analytical data on 1,4-benzodiazepine derivatives, including nitrazepam, concerning the stability of the drug in solution. Nitrazepam is a relatively stable drug at room temperature. However, 2-amino-5-nitrobenzophenone is considered as a decomposition product. The B.P. 1973 (3) describes a method for the detection of this decomposition product, using TLC. Genton and Kesselring (12) have studied the effect of temperature and relative humidity on the stability of nitrazepam in the solid state. The drug and its decomposition products have been determined in a 1% dilution in microcrystalline cellulose. The sample is extracted by shaking with methanol. The extract is chromatographed by TLC on Kieselgel GF 254 using benzene-ethyl acetate - acetic acid (15:9:1) as a developing

solvent. The diffuse reflectance of the spots are measured at 265, 365 and 295 nm for nitrazepam, 2-amino-5-nitrobenzophenone and 3-amino-6-nitro-4-phenyl-4H-quinoline-2-one, respectively.

Meyer, *et al* (13) have published a report on the stability and analysis of the hydrolytic products of nitrazepam. The drug is hydrolysed into 5-nitro-2-aminobenzophenone and 3-amino-6-nitro-4-phenyl-4H-quinoline-2-one (ring contraction). These hydrolytic products can be determined separately by UV absorption after fractionation by TLC on a Kieselgel PF 254 using benzene-isopropanol (9:1) as a solvent; or by means of the absorption of their diazonium salts. Alternatively, the hydrolytic products can also be determined together by means of a (dead-stop) titration with 0.01 N sodium nitrite solution. Meyer *et al* (14) have also studied the effect of pH on the stability of 1,4-benzodiazepine derivatives in injection formulations.

## 5. Metabolism

The metabolites of nitrazepam in man and rat is shown in Fig.5. With the exception of substance IV, which was described by Beyer and Sadee (15), and substance X, which is still hypothetical, the other compounds listed have been proved by Rieder and Wendt (16) to be biotransformation products of the drug appearing in the urine. They have been isolated by various procedures of extraction, column chromatography, and thin-layer chromatography, and their chemical structures have been elucidated by chemical reactions, comparison with authentic samples, mass spectrometry, nuclear magnetic resonance spectrometry, and, in the cases of II and III, also by ultraviolet and infrared spectrometry. The main metabolic pathway in man and rat indicates (Fig.5) the reduction of the nitro group to the corresponding amine II and - by acetylation of II - to the 7-acetamido derivative III, which is the major metabolite. A small proportion of II and III is hydroxylated in position 3, yielding compounds IV and V.

Another metabolic pathway consists of the cleavage of the benzodiazepine ring, with the formation of the benzophenone derivatives VI, VII, and VIII. There is some evidence that the acid X is formed during the generation of the benzophenones, which can be called an opened lactam. The end product of this line, in man, the 2-amino-3-hydroxy-5-nitro-benzophenone VII and, in rat, the 2-amino-5-nitro-4-hydroxy-benzophenone VIII. Part of compound VIII

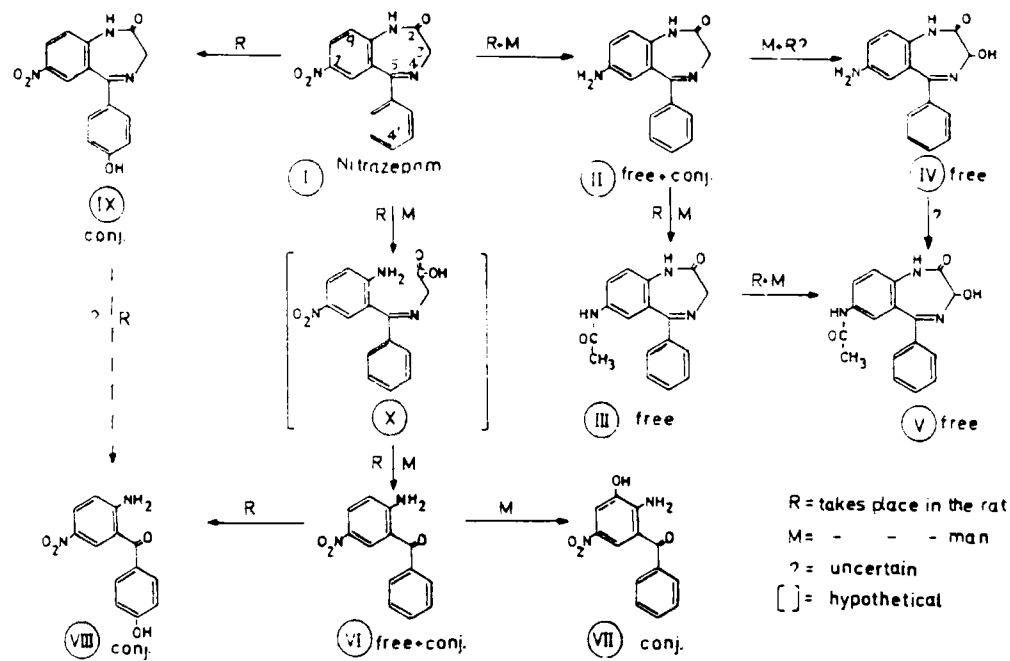


Fig. 5 - Proposed metabolic pathways of nitrazepam in man and rat.

may be possibly derived from the 4-hydroxylated nitrazepam-IX, which has been found in the urine of rat, but not in man (16). The phenolic substances VII, VIII, and IX are excreted almost exclusively; II and VI only to a minor part in conjugated form.

The distribution, excretion and pharmacokinetics of nitrazepam have been discussed by Rieder and Wendt (16).

## 6. Methods of Analysis

### 6.1 Titrimetry

#### 6.11 Aqueous

Blaszek-Bodo, et al (17) have described a diazometric method for the determination of nitrazepam in pure form and pharmaceutical formulations. The method is based on diazotisation reaction in which the drug is first hydrolysed with hydrochloric acid in the presence of zinc to afford 2,5-diaminobenzophenone. This product is titrated against standard sodium nitrite solution. The method proved to be accurate and there is no interference from the drug excipients.

#### 6.12 Non-aqueous

A non-aqueous titration method has been described (3) for the quantitative analysis of nitrazepam as the pure drug. The drug is titrated by perchloric acid in acetic acid and the endpoint is determined potentiometrically.

### 6.2 Spectrophotometry

#### 6.21 Colorimetry

Colorimetric methods have been used for the determination of nitrazepam in various preparations. Wassel and Diab (18) have developed the following procedures for the determination of nitrazepam in pharmaceutical formulations and urine samples:



## a) Ferrous hydroxamate procedure:

To an ethanolic solution (1 ml  $\equiv$  0.2 to 5 mg of nitrazepam), add filtered Goddu reagent [12.5% methanolic hydroxylammonium chloride - 12.5% methanolic sodium hydroxide (1:1)] (3 ml). The solution is heated at 45°C for 50 minutes then cooled and the ferrous reagent  $[(\text{NH}_4)_2 \text{SO}_4 \text{Fe}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}]$  (20 gm) dissolved in 70% aqueous perchloric acid (10 ml) is added. Shake the solution and dilute to 25 ml with acetate buffer solution (0.1 M sodium acetate adjusted to pH 1.5 with 70% aqueous perchloric acid). Measure the extinction at 550 nm and obtain the amount of nitrazepam by reference to a calibration graph, which is rectilinear from 0.1 to 5 mg of nitrazepam.

Treat urine samples (200 to 400 ml) with ammonia (to pH 10) and extract nitrazepam and its metabolites with chloroform (4x100 ml). Wash the combined extracts with water, add anhydrous ethanol (5 ml) and evaporate to dryness in vacuo at 50°. Dissolve the residue in anhydrous ethanol (5 ml), add the Goddu reagent (3 ml) and continue as above.

## b) Citric acid method

To the ethanolic solution (1 ml  $\equiv$  up to 50  $\mu$ g of nitrazepam), add 5 ml of citric acid reagent [citric acid (2 gm) dissolved in ethanol (10 ml) and anhydrous acetic acid (90 ml)] and heat the solution at 70-80°C for 20 minutes. Dilute the solution to 25 ml with ethanol and measure the extinction at 510 nm. This procedure is suitable for routine analyses.

Diab (19) has developed a colorimetric method for the analysis of the drug in formulations and its metabolites in blood and urine. The method depends on the color reaction of Porter (20) for the

aromatic nitro compounds. The method is essentially as follows: Transfer 5 ml of standard solution (2 mg of nitrazepam in 100 ml either dimethylformamide or acetone) into separate test-tubes. Add 0.1 ml of 10% tetraethylammonium hydroxide solution to the dimethyl formamide solution or 0.1 ml of 10% sodium hydroxide solution to the acetone solution, shake the mixtures and measure the extinctions at 410 nm. The color formed by either reaction is stable for more than two hours. For the assay of tablets, a quantity of powdered sample is extracted with ethanol. The combined filtered ethanol extracts are diluted to a certain volume and 1 ml of the solution is evaporated to dryness on a steam bath. The residue is dissolved in either dimethylformamide or acetone and proceed as for the standard solution. For samples of blood and urine, nitrazepam is extracted with benzene and determined as above. Metabolites are determined with 4-dimethylaminobenzaldehyde reagent (0.125 gm in 100 ml of 65% sulphuric acid plus 0.1 ml of 5% aqueous ferric chloride solution) and measurement of the extinction at 420 nm.

Raber and Gruber (21) have described a photometric method for estimation of nitrazepam and other 1,4-benzodiazepine derivatives. Nitrazepam is hydrolysed with hydrochloric acid and the resulting 2-aminobenzophenone derivative is diazotised and coupled with 1-naphthol. the extinction of the solution is then measured at 607 nm. The method is applicable for estimation of a mixture of nitrazepam and other derivatives. Also, the procedure is applicable for the determination of nitrazepam in tablet or capsule formulations

Beyer and Sadee (15) have essentially applied the same principle used before for the determination of nitrazepam (and other 5-phenyl-1,4-benzodiazepines) and for investigations on the metabolism of nitrazepam. The diazonium salt being treated with 2% sulphamic acid solution and the diazo compound is then coupled with N-1-naphthylethylenediamine dihydrochloride. The extinction of the resulting azo dye is measured at 533 to 535 nm and referred to calibration graphs.

Recently, Blaszek-Bodo, et al (22) have reported a method for estimation of nitrazepam. The drug is hydrolysed and simultaneously reduced and the resulting 2,5-diaminobenzophenone is diazotised and coupled with N-1-naphthylethylenediamine.

Egg (23) has devised a qualitative method for the detection of nitrazepam and other derivatives by the colour reaction of Sawicki and Johnson. Benzodiazepines can be detected after TLC separation by spraying the chromatogram with 1% 2,5-dimethoxytetrahydrofuran solution in acetic acid and drying for 5 to 10 minutes at 100°C; a reddish-violet spot is produced on re-spraying with 2% 4-dimethylaminobenzaldehyde solution in acetic acid - conc. hydrochloric acid (17:3). The sensitivity of the colour reaction is dependent on the substituents in the benzodiazepine molecule.

#### 6.22 Spectrofluorimetry

This method has been used for the identification in urgent toxicological analysis of 1,4-benzodiazepines used in therapeutic treatment (24). The gastric fluid is made neutral or weakly acid and the drug is extracted by ether. The extract is evaporated to dryness and the residue is then dissolved in  $\text{HClO}_4$ ,  $\text{H}_3\text{PO}_4$  or  $\text{H}_2\text{SO}_4$ .

### 6.23 Ultraviolet

The determination of nitrazepam and other benzodiazepines in solutions, injections, tablets and syrups pharmaceutical formulations by UV spectrophotometry has been described (25). Nitrazepam is determined at 259 nm in neutral 96% ethanol.

Another report (26) has been published for the spectrophotometric determination of nitrazepam in methanolic solution at 259 and 309 nm in concentration ranges 0.2 to 2 and 0.4 to 3 mg dl<sup>-1</sup>, respectively.

The B.P. 1973 (3) describes a method for the assay of nitrazepam tablets depending on acid hydrolysis and the resulting 2-amino-5-nitrobenzophenone is measured spectrophotometrically at a maximum of about 280 nm ( $E_{1cm} 1\% = 910$ ).

## 6.3 Chromatography

### 6.31 Thin Layer Chromatography

A compilation of qualitative colour and precipitation reactions, spectrophotometric and TLC data (useful for identification purposes and for quantitative assay methods) related to nitrazepam has been reviewed by Dobrecky, *et al* (27). Several Reports had been published concerning the chromatographic identification and separation of nitrazepam and its metabolites as shown in Table 2.

Table 2

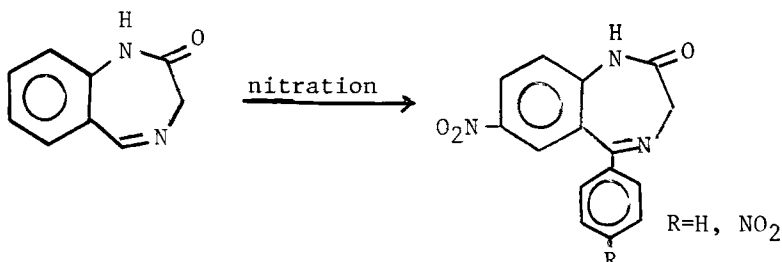
Solvent System	Absorbent	Detection	Reference
Toluene-acetone-conc. aq. ammonia (50 : 50 : 1)	Kieselgel GF254	-UV at 254 or 356 nm -diazotisation and coupling	28

Solvent System	Absorbent	Detection	Reference
Ethyl acetate-propa- nol-diethyl-amine. (70 : 30 : 1)  Methanol-1,2-dichlo- roethane-conc. aqu. ammonia (10 : 90: 1)		-reduction by $\text{Na}_2\text{S}_2\text{O}_4$ to give coloured derivative	
Toluene-diethylamine (4 : 1)  Ethyl acetate-metha- nol-acetic acid (90 : 10 : 1)	Kieselgel $\text{GF}_{254}$  Silica gel	-UV at 254 nm  -UV at 254 nm	29  30
Heptane-chloroform- ethanol (5 : 5 : 1)  Ethyl acetate-1,2- dichloroethane-25% aq. ammonia (8 : 2 : 1)	Silica gel $\text{GF}_{254}$	-UV at 254 nm -Spraying with conc. $\text{H}_2\text{SO}_4$ , $\text{HCl}$ $\text{H}_3\text{PO}_4$ , $\text{HClO}_4$ & the color of the fluorescence in radiation at 254 and 366 nm is noted.	31
Dioxane-benzene- hexane-conc. aq. ammonia  (9 : 10 : 14 : 1)  chloroform-acetone- tetrahydrofuran ( 9 : 1 : 1)	Kiesel gel $\text{GF}_{254}$	-fluorescence  -spray with Drag- endorff reagent -1% 2-furaldehyde solution in acetone solution of 10 gm $\text{H}_2\text{SO}_4$ in 90 ml acetone	32

Solvent system	Absorbent	Detection	Reference
Shellsol A -methanol- 25% aq. ammonia (85 : 15 : 1)	Silica gel G	-Dragendorff reagent diluted 1:10 with 10% HCl	33
Chloroform-benzene- ether-tetrahydrofur- an-acetone-acetic acid (35: 15: 16:10: 5:3)	Silica gel	-Photometrically (UV at 230-300 nm)	34
Chloroform-ether (3:2)	Silica gel G	-UV at 254 nm and sprayed with ethanolic 0.01% N-1-naphthyl- ethylenediamine	35
Chloroform-toluene- ethanol (20 : 30: 1)	Merck Aluminium oxide F <sub>254</sub> (type E)	-UV at 254 nm -Spraying with K <sub>2</sub> PtI <sub>6</sub>	
Chloroform-ethanol	Whatman SG 81		36
Benzene-chloroform (3:1) Chloroform-ethanol (29:1)	Aluminium oxide F <sub>254</sub>	-UV at 254 nm  -Fluorescent spots at 366 nm -diazotisation followed by spray- ing with 0.1% aq. N-1-naphthyl-NN- diethylpropane-1, 2-diamine hydro- chloride and heat at 50°C.	24

Negritescu *et al* (37) have described a TLC separation method of the reaction products formed during synthesis of nitrazepam by nitration of 2,3-dihydro-5-phenyl-1H-1,4-

benzodiazepine-2-one. Nitrazepam is formed along with a dinitro derivative. The latter



can be separated from the reaction mixture by TLC on Kieselgel H, using 6 solvent systems, namely:

- 1) benzene-n-butanol-formic acid (50:28:8)
- 2) dibutyl ether-ethyl acetate-formic acid (25:75:8)
- 3) benzene-ethyl acetate - formic acid (25:75:5 ; 25:75:10; 25:75:15; 25:75:20)

After drying the chromatograms are sprayed with HNO<sub>3</sub> (0.15 ml of conc. HNO<sub>3</sub> in 10 ml of ethanol) and the plates are then examined under UV radiation.

Schuetz (38) has developed a chromatographic method for the detection of nitrazepam and its major metabolites. The sample (e.g. urine extract) is subjected to TLC, with benzene-isopropyl alcohol-25% aq. ammonia (80:20:1) as the solvent. The drug and its metabolites, are then hydrolysed and reduced by spraying with acidic TiCl<sub>3</sub> solution. The plate is treated with gaseous ammonia to convert any amine salts into the free bases. A second development at right angles with the same mobile phase is then carried out. Diazotisation followed by coupling with N-1-naphthylethylenediamine makes it possible to detect amounts as low as 0.02 ug per spot.

### 6.32 Column Chromatography

Golovenko, *et al.* (39) have reported a method for the separation of nitrazepam and its metabolites from rat urine. Portions (10 to 100 ug each) of nitrazepam and its possible metabolites, dissolved in chloroform-hexane (1:1), are applied to a column (10 cm x 0.5 cm) of KSK-1 Silica gel (76 mesh) and the column is washed with 10 ml of hexane; clean separation are obtained by stepwise change of eluent. The collected 1-ml fractions are evaporated in vacuum, each residue is dissolved in 4 ml of ethanol, and the absorbances of the resulting solutions are measured at the appropriate wavelengths. The order of elution of compounds investigated in model mixtures and eluents used (as 10-ml portions) are as follows:

Nothing eluted in  $\text{CCl}_4$ ; nitrazepam, hexane-acetone (4:1); 7-amino-1,2-dihydro-5-phenyl-3H-1,4-benzodiazepin-2-one, chloroform-acetone (4:1); and 7-acetamido-1,2-dihydro-5-phenyl-3H-1,4-benzodiazepin-2-one, chloroform-acetone (4:1).

Sawada, *et al* (30) have described a method for the isolation and identification of nitrazepam and its metabolites in rabbit urine. The method involves the sorption on a column of XAD-2 resin, which is eluted by methanol and ethyl acetate-methanol-acetic acid (90:10:1). Conjugated metabolites in the eluate from the column are hydrolysed enzymically, and the liberated compounds are extracted into ethyl acetate.

Missen (40) has reported a procedure for extracting nitrazepam from the blood, by column chromatography. The procedure involves the absorption of the drug on activated charcoal, Amberlite XAD-2 resin and Celite eluting with chloroform.

### 6.33 High Performance Liquid Chromatography

Moore, *et al* (41) have reported a HPLC method for the analysis of nitrazepam and its meta-



bolites, in urine. The urine sample is adjusted to pH7 with acetate buffer solution and extracted with ethyl acetate. The combined extracts are evaporated to dryness and the residue is dissolved in ethyl acetate. Portions of this solution are subjected to HPLC on a stainless steel column (50 cm x 2mm) packed with Zipax SAX (30  $\mu$ m) and operated with hexane-ethyl acetate (7:3) as mobile phase, at a rate of 1 ml per minute, and detection at 260 nm. This method is suitable for the determination of the drug and of its 7-amino- and 7-acetamido-metabolites up to 700 ng of each injected. Detection limits range from 20-100 ng and the recovery of the added compounds is 80%.

Harzer and Barchet (42) have described a method for the analysis of nitrazepam and other benzodiazepines and their hydrolysis products, namely; benzophenones, by reversed-phase HPLC. The method is applied to the analysis of extracts from blood and urine. The method is based on the separation, by HPLC on a column (25 cm x 4 mm) of LiChrosorb SI-100 (grain size 10  $\mu$ m), operated at room temperature and 750 p.s.i. with aqueous methanol (60 to 100% of methanol) as the mobile phase at the rate of 0.75 ml per minute. Another procedure has been reported (43) for the analysis of benzodiazepines, including nitrazepam, and their metabolites, by enzymic digestion and high-performance liquid chromatography. The procedure involves the liberation and extraction of the drugs and/or metabolites, with ether. The ether extract is dried and evaporated and the residue is dissolved in anhydrous ethanol. Few microlitres of the ethanolic solution are submitted to HPLC on a column (150 mm x 4.6 mm) packed with Spherisorb-5-ODS and operated with 0.025 M  $\text{Na}_2\text{HPO}_4$ -methanol (2:3), adjusted to pH 7.8, as mobile phase, at a rate of 1 ml per minute. The detection is done by UV spectroscopy at 254 nm.

6.34 Gas Liquid Chromatography

GLC has been extensively used as a method for the determination of nitrazepam in pharmaceutical preparation; also for the determination of the drug and its metabolites in biological fluids and tissues. Furthermore, GLC is one of the most convenient methods for the detection and determination of nitrazepam in toxicological screening. The drug is chromatographed without derivatisation or after acid hydrolysis into 2-amino-5-nitrobenzophenone. The gas liquid chromatographic conditions are given in Table 3.

Table 3

Stationary phase	Detector	Carrier gas	Column temperature, °C	Remarks*	Reference
2% of OV-17 on Chromosorb G-Hp	Flame ionisation	N <sub>2</sub>	260	-	44
3% of OV-1 on Chromosorb Q (60 to 80 mesh)	Flame ionisation	N <sub>2</sub>	245	-	45
3% of OV-1 on Chrom Q (100 to 120 mesh)	Flame ionisation	N <sub>2</sub>	250 210	For the drug & its metabolites For the hydrolysis products	46
3% of OV-17 on Diatomate CQ (80 to 100 mesh)	<sup>63</sup> N elec-tron capture	Ar-CH <sub>4</sub> (9:1)	245	After acid hydrolysis	47
3% of OV-17 or Sp-2250 on chromosorb W or Supelcoport (100 to 120 mesh)	<sup>63</sup> Ni elec-tron capture	N <sub>2</sub>	245 275	as hydrolytic product -	48

Stationary phase	Detector	Carrier gas	Column temperature, °C	Remarks*	Reference
3% of OV-17 on Gas-Chrom Q (60 to 80 mesh)	$^{63}\text{Ni}$ electron capture	Ar	235	after acid hydrolysis	49
3.8% of SE-30	Flame ionisation	He	240	after acid hydrolysis	50
2% of OV-17 on Chromosorb W.H.P. (80 to 100 mesh)	$^{63}\text{Ni}$ electron capture	He	275	-	51

\* Unless otherwise stated in the remarks, the drug has been determined underivatized.

Lafargue, et al (24) have reported a gas chromatographic method for the identification of nitrazepam in the gastric fluid. The latter is extracted after being made neutral or weakly acidic, with ether. The extract is then examined by GLC in a 2-metre column packed with 3% of OV-17 on Gas Chrom Q (100 to 120 mesh) and operated at  $250^{\circ}$  (or  $210^{\circ}$  for the hydrolysis products).

#### 6.4 Polarography

Several methods have been published for the determination of nitrazepam and related derivatives in pharmaceutical formulations as well as in biological fluids, (blood, urine, and serum).

Oelschlaeger, et al (52) had reported a procedure for the determination of nitrazepam after the drug is separated by a TLC on sheets having silica gel or alumina as adsorbent on a polyethylenetetraphthalate backing without removal of the plastic foil

The plastic foil is stable to all solvents used and the binder and the sorbent do not interfere with the development of the current versus potential curve. Any zinc-containing impurities do, however, interfere and must be masked with EDTA before measurement. A dropping-mercury electrode is used in the determination in which nitrazepam and its 7-amino reduction product are determined. Dimethyl sulfoxide and dimethylformamide are used as solvents. The recovery is more than 95%. For the amino compound, good recovery is achieved only if the sorbent is removed; this is not necessary for nitrazepam.

Ellaithy, *et al* (53), had reported the determination of some benzodiazepines, among which nitrazepam is included, by differential pulse polarography with a dropping-mercury indicator electrode and a saturated mercuric sulphate reference electrode. The calibration graph of peak current versus drug concentration is rectilinear for concentration down to 0.14 ug per ml. Nitrazepam is dissolved in acetonitrile and the solution is buffered at pH 4.8. The method is applicable for the determination of nitrazepam and some other benzodiazepines in urine (2 ml) without prior extraction.

Halvorsen, *et al* (54) have reported the electro-reduction and polarographic determination of nitrazepam in serum. The electro-reduction of nitrazepam has been studied by polarography, cyclic voltammetry, chromopotentiometry and controlled-potential coulometry. In a phosphate buffer solution of pH 6.9 there are two reduction steps; the first giving a well-defined polarographic wave being a four-electron reduction of the nitro-group and the second being a two-electron reduction. The oxidised form of nitrazepam is strongly adsorbed on the electrode surface and thus it is possible to determine nitrazepam in the presence of proteins.

The polarographic determination of nitrazepam in whole blood, in acute poisoning, has been reported (55). The procedure is based on administration of nitrazepam to rats and the homogenised blood samples are diluted with an electrolyte consisting of 1:1 mixture of methanol with Britton-Robinson buffer solution of pH 2.2 to 3.3. The solutions are examined polarographically in the range 0.0 to 0.6 V.

The polarographic and spectral behaviour of 7-amino and 7-acetamido nitrazepam metabolites have been utilised to effect separations of mixtures (56). Changes of UV absorption spectra with pH in solution are used to determine  $pK_a$  values for nitrazepam metabolites. 7-Acetamido-nitrazepam gives two  $pK_a$  values, corresponding to protonation in acid and deprotonation of the neutral molecule in alkaline media. 7-Amino nitrazepam gives three  $pK_a$  values, the third one being due to additional protonation in acid media. The spectra are explained by considering them to be superimposed spectra of the two benzene rings, one monosubstituted, and one trisubstituted within the molecule. Differences in the  $pK_a$  values or the polarographic behaviour between nitrazepam and its metabolites are used to effect novel separation after solvent extractions from aqueous buffered solutions.

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# NITROGLYCERIN

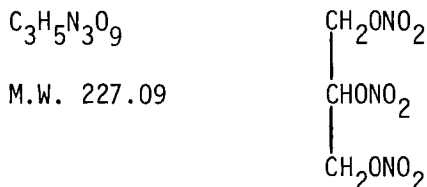
*Edward F. McNiff, Peter S. K. Yap, and  
Ho-Leung Fung*

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## 1. Description

### 1.1 Name, Formula, Molecular Weight

Nitroglycerin (glyceryl trinitrate, trinitroglycerol) is 1,2,3-propanetriol trinitrate.



### 1.2 Appearance, Color, Odor

Pale yellow, odorless, oily liquid with a sweet, burning taste.

## 2. Physical Properties

### 2.1 Nuclear Magnetic Resonance Spectrum

An NMR spectrum of nitroglycerin is shown in Fig. 1. The sample was isolated from a lactose adsorbate by ether extraction. After solvent evaporation, the sample was purified by hexane elution from a neutral silica column, followed by gentle hexane evaporation under a stream of nitrogen. A  $\text{CDCl}_3$  solution spectrum was run on a Varian T-60A spectrometer using trimethylsilane as the internal reference. The multiplet at  $\sim 4.8 \delta$  is assigned to the four protons at the 1 and 3 carbon atoms, and that at  $\sim 5.5 \delta$  is assigned to the proton at the C-2 position. Small singlets appearing at  $\sim 1.5 \delta$  and  $\sim 7.2 \delta$  are apparently due to trace impurities of hexane residue and non-deuterated chloroform, respectively. The integrated areas correlate well with the structural assignments. No attempts were made to interpret the splitting patterns.

### 2.2 Infrared Spectrum

The IR spectrum, Fig. 1, was obtained on a Perkin-Elmer model 272B infrared spectrophotometer. Nitroglycerin was isolated and purified as described in 2.1. Sample mounting was by formation of a capillary film of neat nitroglycerin between NaCl plates. The band at  $850 \text{ cm}^{-1}$  is found in all organic and inorganic nitrates. Bands at  $1650$  and  $1280 \text{ cm}^{-1}$  are attributed to symmetrical stretching and deformation vibrations of the  $\text{NO}_2$  group, respectively. This spectrum compares well with one that was reported along with a large number of other nitrates by Pristera *et al.*<sup>1</sup>.

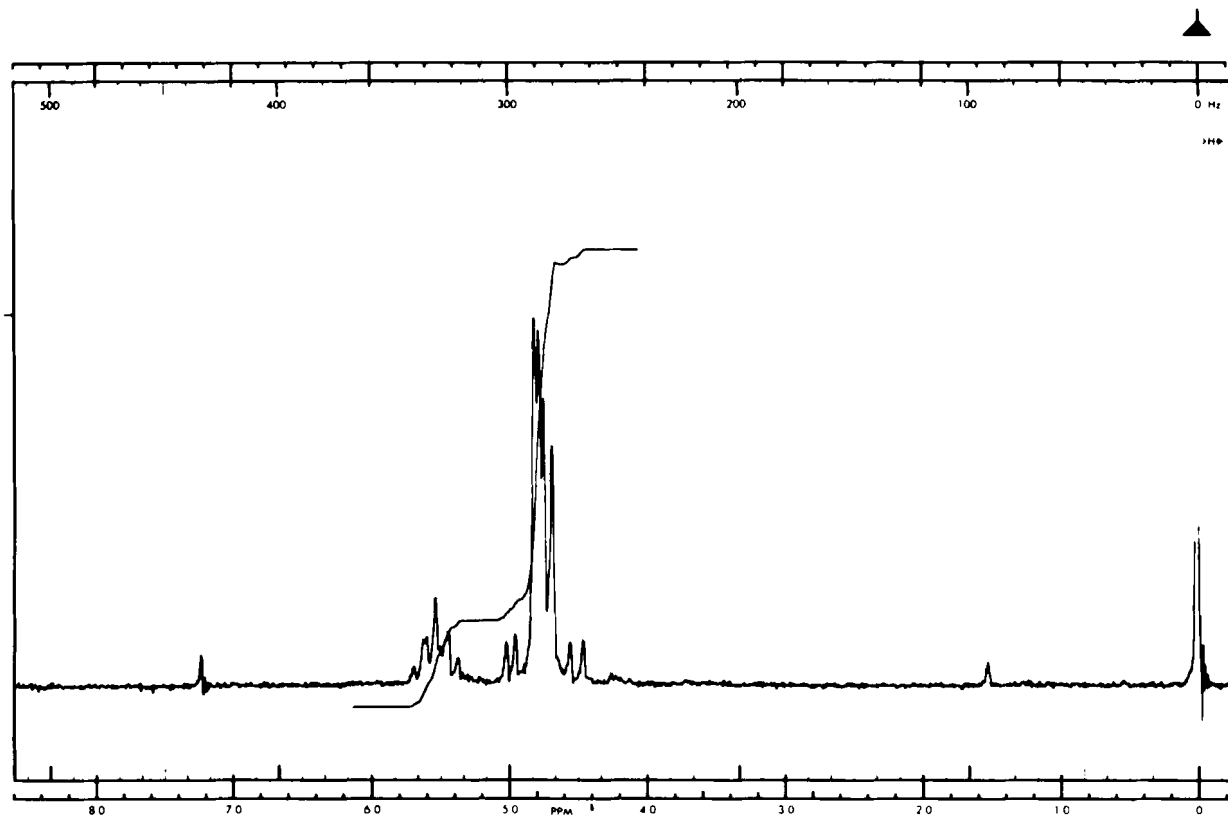


Fig. 1: NMR Spectrum of nitroglycerin

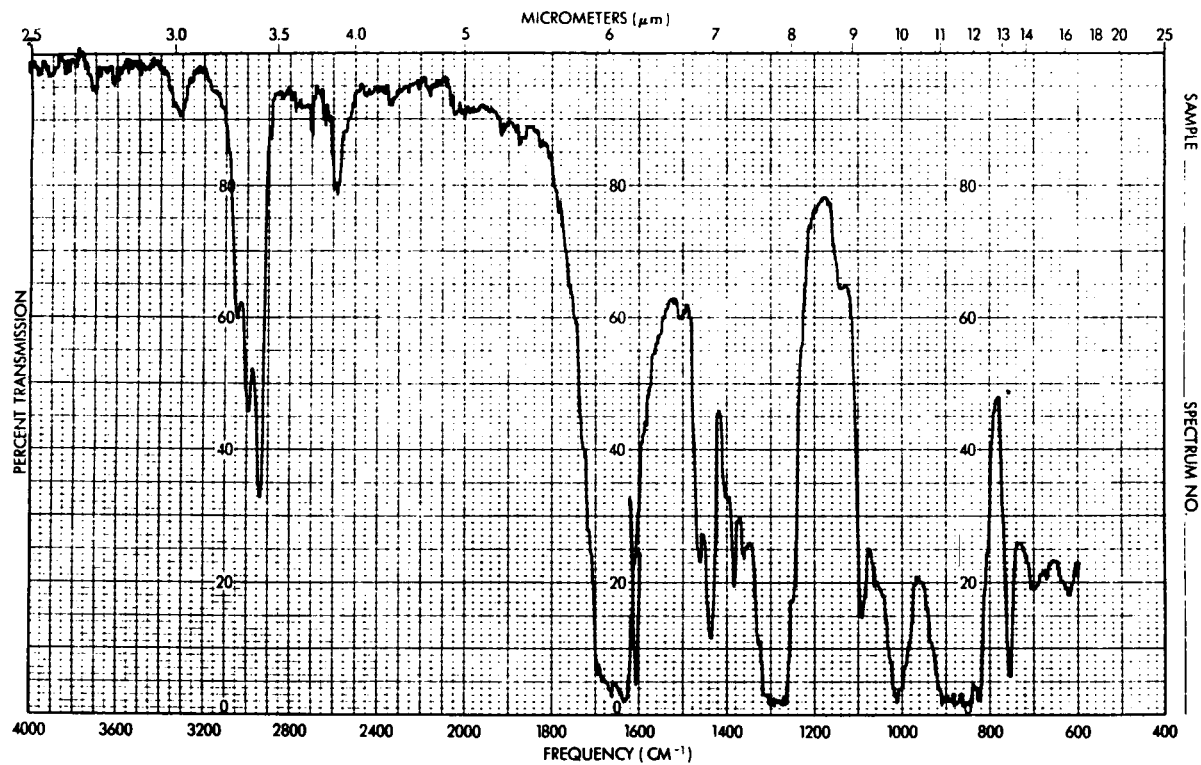


Fig. 2: IR Spectrum of nitroglycerin

### 2.3 Ultraviolet Spectrum

Nitroglycerin, in a solution of neutral pH, has no appreciable absorbance in the near ultraviolet and visible region<sup>2</sup>.

### 2.4 Mass Spectrum

The mass spectra of 21 nitrate esters, including nitroglycerin, were run on an A.E.I. MS2H single focusing mass spectrometer, operating at 70 eV<sup>3</sup>. The base peak of M/e 46 ( $\text{NO}_2^+$ ) is characteristic of the lower nitrates. Other major peaks, with their corresponding structural assignments, are shown in Table I:

TABLE I<sup>3</sup>

Mass Spectral Characteristics of Nitroglycerin

<u>M/e</u>	<u>Relative Intensity</u>	<u>Structural Assignment</u>
28	6	$\text{CO}^+$
29	15	$\text{CHO}^+$
30	24	$\text{NO}^+$
43	5	$^+\text{OCH}\cdot\text{CH}_2$
46	100	$\text{NO}_2^+$
76	9	$\text{CH}_2\cdot\text{O}\cdot\text{NO}_2^+$

### 2.5 Vapor Pressure and Boiling Point

The vapor pressure of nitroglycerin at 20<sup>o</sup>, 25<sup>o</sup> and 37<sup>o</sup> has been reported<sup>4,5</sup> as  $2.6 \times 10^{-4}$ ,  $5.5 \times 10^{-4}$  and  $2.2 \times 10^{-3}$  Torr, respectively. The gravimetric Knudsen effusion technique has been used to study the vapor pressure of nitroglycerin in molded tablets<sup>6</sup>.

Pure nitroglycerin has an apparent boiling point of 145<sup>o</sup> C (with violent decomposition)<sup>7</sup>.

### 2.6 Melting and Crystal Properties

At low temperatures, nitroglycerin exists in two crystal forms. It freezes to form a stable dipyramidal polymorph which melts at 13.2<sup>o</sup> C. Under some conditions, an unstable triclinic crystal (m.p. 2.2<sup>o</sup> C) may form. This labile polymorph will convert into the more stable form upon standing<sup>1</sup>.

### 2.7 Density

The density of nitroglycerin is 1.601 at 15<sup>o</sup> C<sup>4</sup>.

### 2.8 Viscosity<sup>7</sup>

<u>Viscosity (cP)</u>	<u>Temperature (°C)</u>
35.5	20
21.0	30
9.4	50
6.8	60

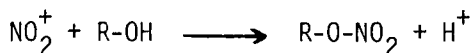
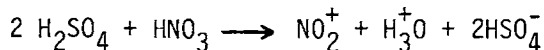
### 2.9 Solubility

The following information is available from reference 7: nitroglycerin has an aqueous solubility of 1.73 and 2.46 mg/ml at 20° and 60° C respectively; ethanol dissolves nitroglycerin to the extent of 375 mg/gm at 0° and 540 mg/gm at 20°; hot ethanol is miscible with nitroglycerin in all proportions; other solvents completely miscible with nitroglycerin are: acetone, ether, glacial acetic acid, ethylacetate, benzene, toluene, phenol, nitrobenzene, chloroform, ethylene chloride and nitric esters. Additionally, it has been reported<sup>4</sup> that nitroglycerin is miscible with pyridine and ethylene bromide, but is only sparingly soluble in petroleum ether, liquid petrolatum and glycerol. The solubility in methanol and carbon disulfide is 56 mg/gm and 8.3 mg/gm respectively.

Using the aqueous solubility of nitroglycerin and partitioning data, Horhota and Fung<sup>8</sup> calculated nitroglycerin solubility in different water-polyethylene glycol 400 co-solvent systems. For instance, the calculated solubility of nitroglycerin in a 90% (w/v) polyethylene glycol 400-water mixture was estimated at 135 mg/ml.

### 3. Synthesis

Organic nitrate synthesis is commonly accomplished by esterification of the corresponding alcohol<sup>7,9,10</sup>. In the case of nitroglycerin, the nitrating mixture consists of equal volumes of nitric and sulfuric acids. A small amount of urea or urea nitrate is added as a scavenger for any excess nitrous acid present. Esterification is carried out by slow addition of glycerol to the mixed acids.



Careful control of temperature and rate of addition reduces or eliminates the side reaction of alcohol oxidation. The ester can be separated by pouring the reaction mixture into cold water or by careful distillation.

## 4. Stability

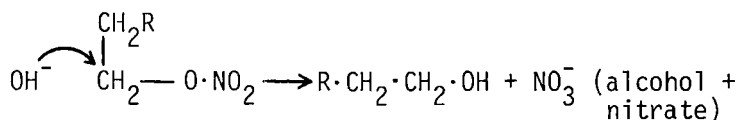
### 4.1 Chemical Stability

#### 4.11 Hydrolysis

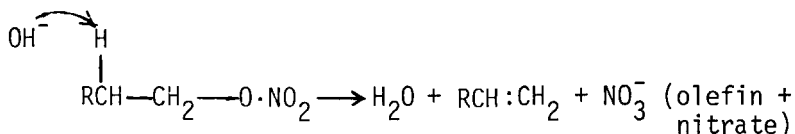
The stability of nitroglycerin in alcoholic solutions as a function of pH has been studied by Amshler<sup>11</sup>. The compound is relatively stable in neutral and weakly acidic solutions but degrades very rapidly in the presence of alkali<sup>12,13</sup>.

Alkaline hydrolysis of nitrate esters can proceed via three possible mechanisms<sup>14</sup>:

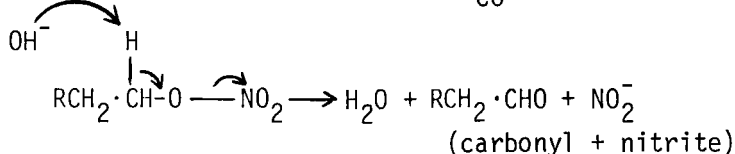
(a) Nucleophilic substitution ( $S_N2$ )



(b)  $\beta$ -hydrogen elimination ( $E2$ )



(c)  $\alpha$ -hydrogen elimination ( $E_{CO}2$ )



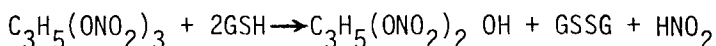
The initial step of alkaline hydrolysis of nitroglycerin involves  $\alpha$ -elimination at the secondary nitrate group resulting in the formation of nitrite ion and a carbonyl. This electronegative carbonyl group causes either of the remaining primary nitrates to be more susceptible to nucleophilic attack. A slower reaction on the primary nitrate, producing the alcohol and nitrate ion, becomes more important with increasing ratios of hydroxide ion to nitroglycerin<sup>2</sup>. Alkaline degradation of nitroglycerin is accompanied by the appearance and subsequent disappearance of an ultraviolet absorption peak near 335 nm due, presumably, to the monocarbonyl intermediate. The maximum absorbance and the peaking time of this chromophore are dependent upon initial concentrations of nitroglycerin and hydroxide ion. This reaction is the basis for a kinetic



assay procedure for nitroglycerin<sup>15-17</sup> which is discussed later.

Acid catalyzed hydrolysis of nitroglycerin was found to occur at a much slower rate than that of alkaline hydrolysis<sup>14,18</sup>. Incubation of nitroglycerin at 37° for 15 minutes in 4 N NaOH resulted in essentially complete denitration, while in 4 N HCl, nitroglycerin was degraded only 28% after 6 hours<sup>18</sup>. Under acid conditions, twice as much glyceryl-1,2-dinitrate is formed compared to glyceryl-1,3-dinitrate<sup>19</sup>, suggesting that the initial reaction site is on the primary nitrate. The kinetics of nitroglycerin hydrolysis in nitric acid at 20° to 80° C has also been studied<sup>20</sup>.

Klason and Carlson<sup>21</sup> observed that alkaline degradation of nitroglycerin in the presence of phenylmercaptan resulted in the formation of diphenyldisulfide and glycerol. It was later shown that reduced glutathione (GSH) reacts with nitroglycerin to produce inorganic nitrite ions<sup>22</sup>. Subsequent studies<sup>23,24</sup> characterized the reaction as:



This reduction process was found to be relatively slow. With equal and 10 molar equivalents of GSH, 0 and 22% of nitroglycerin were degraded within 1 hour at 37° C, respectively. Biotransformation of nitroglycerin in the body is apparently closely related to the above reaction. The *in vivo* process, however, is a much faster reaction because it is enzymatically catalysed.

#### 4.12 Photolytic and Thermal Stability

Although it was suggested that nitroglycerin is susceptible to photolysis<sup>25</sup>, there is no supporting evidence in the literature. In aqueous solution, exposure to light does not lead to accelerated disappearance of nitroglycerin<sup>26</sup>.

The thermal decomposition of nitroglycerin is highly dependent on the ratio of nitroglycerin mass to the volume of the reaction vessel<sup>27</sup> presumably due to product inhibition by NO<sub>2</sub>. Within the temperature range of 140° to 160° and a mass to volume ratio of 3.5 x 10<sup>-3</sup> gm cm<sup>-3</sup>, vapor phase degradation follows first order kinetics and obeys the Arrhenius relationship with an energy of activation (E<sub>a</sub>) of approximately 36 kcal/mole. Deviation from first order kinetics is observed in the liquid phase, and is probably due to autocatalytic effects<sup>27</sup>. Below 140°, the decomposition reactions are also affected by autocatalysis<sup>28</sup>.

## 4.2 Physical Stability

Instability of nitroglycerin in pharmaceutical dosage forms can generally be attributed to two processes, viz: (a) volatilization leading to loss of drug to the atmosphere, and (b) sorption of drug to plastics. The appreciable volatility of nitroglycerin at room temperatures has been shown to be a major cause of loss of potency and inter-tablet migration of drug during storage of unstabilized sublingual tablets<sup>6,29</sup>. This problem has been somewhat alleviated by the addition of polyethylene glycol 400 and povidone as stabilizers<sup>30-34</sup>. Drug loss due to sorptive phenomena has been implicated when nitroglycerin tablets are stored in plastic containers and unit dose strip packages<sup>34,35</sup>. FDA regulations (promulgated in 1972)<sup>36</sup> require that nitroglycerin tablets be packaged in tight containers, preferably of glass with metal screw caps, and dispensed in the original, unopened container with a special warning label. No more than 100 tablets should be dispensed in each container.

Problems of stability and potency relating to extemporaneously prepared nitroglycerin infusions have recently been pointed out<sup>37</sup>. Extensive loss of nitroglycerin from intravenous solutions stored in plastic i.v. bags can be attributed to sorption<sup>26,37-42</sup>, since intact drug can be recovered from the container<sup>40</sup>. Plastic tubing used for the administration of intravenous nitroglycerin solutions also causes drug loss due to sorption<sup>38,43</sup>. High density polyethylene tubing, however, is non-adsorptive<sup>43</sup>.

## 5. Metabolism

The metabolism of nitroglycerin and other organic nitrates has been extensively reviewed<sup>44-46</sup>. Only a summary of the major findings regarding the metabolism of nitroglycerin is presented here.

### 5.1 Biochemistry

Heppel and Hilmo<sup>22</sup> showed that the spontaneous reaction between nitroglycerin and GSH to be catalyzed by a hog liver microsomal enzyme. Initial characterization of the enzymatic process using partially purified hog liver acetone powder demonstrated that the system is anaerobic, has an optimal pH of 7-8, is inhibited by cupric sulfate and stimulated by cyanide. Subsequent investigations showed that the liver enzymes, purified from rat and guinea pig liver and named organic nitrate reductases (ONR), consisted of 2 distinct fragments with different activity for nitroglycerin and other organic nitrates<sup>47,48</sup>. The two different enzymes were estimated to have a molecular weight of 14,000 and

43,700 respectively.

Needleman and Hunter<sup>23</sup> developed a rapid and sensitive enzymatic assay to quantify the relative activities of rat liver ONR toward different organic nitrates. This assay measures the disappearance of reduced triphosphopyridine nucleotide (TPNH), which is consumed for the production of GSH, which in turn is required for the denitration of the organic nitrate (Fig. 3).

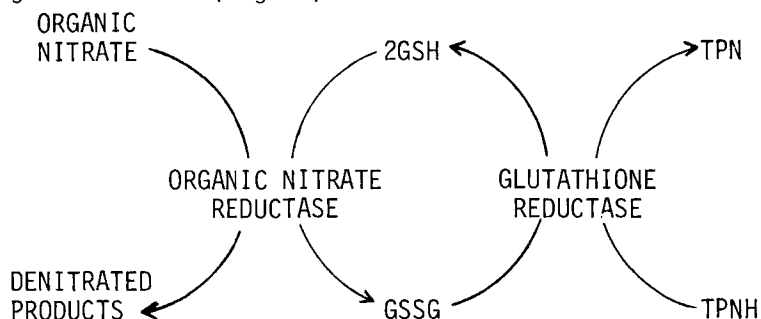


Figure 3. Biochemical reactions involved in the denitration of organic nitrates.

The maximum velocities of the enzymatic reaction of different organic nitrate has been reported<sup>23</sup>. Polynitric esters are rapidly metabolized by liver ONR, in the order of mannitol hexanitrate >> erythritol tetranitrate >> nitroglycerin. Replacement of a nitrate group with a hydrogen atom or a hydroxy group, or introduction of an ether linkage into a linear chain nitrate compound, decreases the rate of enzymatic transformation. Branched chain alcohol nitrates are also significantly less susceptible to organic nitrate reductase degradation. *In vitro* studies have also demonstrated that the metabolism of nitroglycerin in liver homogenates can be enhanced or depressed upon pretreatment of the experimental animals with barbiturates and bromobenzene respectively<sup>49,50</sup>. It was suggested<sup>51</sup> that phenobarbital pretreatment caused increase in the amount of reductase enzyme as well as the activity of GSH generating capacity.

## 5.2 Site of Metabolism

Needleman and Harkey<sup>49</sup> compared the rate of degradation of nitroglycerin in isolated perfused rat liver to the *in vivo* biotransformation rates. The *in vitro* half time of 2 minutes was comparable to that observed in intact experimental animals. In eviscerated rats, the biological half-

life of nitroglycerin was 7 to 8 minutes as compared to less than 2 minutes in controls<sup>52</sup>. These experiments clearly establish the importance of hepatic metabolism of nitroglycerin in experimental animals. Recently, Maier et al<sup>53</sup> showed that a relationship exists between *in vivo* nitroglycerin bioavailability (100 mg/kg orally in rats) and the *in vitro* liver ONR activity in individual animals. Glutathione-dependent ONR activity was found only in trace quantities in the kidney and was absent in the lung, small intestine, heart and skin. These data suggested that first-pass metabolism of orally administered nitroglycerin occurred primarily in the liver.

Under physiological conditions, rat serum also hydrolyzed nitroglycerin to dinitrates and mononitrates, but at a much slower rate. The half-life of serum degradation was found to be 15 to 20 minutes at 37° C<sup>24,54</sup>. The effects of concentration, temperature, red blood cell hemolysis and silver nitrate addition on nitroglycerin stability in human and rat plasma have also been examined<sup>55</sup>. Within the temperature range of -20° to 37° C, degradation was shown to obey the Arrhenius relationship with an apparent energy of activation (Ea) of 24.1 and 19.0 kcal/mole for human and rat plasma, respectively. Depending upon the temperature, nitroglycerin is degraded 10-50 times faster in rat plasma compared to human plasma.

Hepatic and blood metabolism of nitroglycerin has been demonstrated in other animal species<sup>56</sup>. Human liver biopsy samples were shown to contain a glutathione dependent ONR capable of rapid biotransformation of nitroglycerin to its lower nitrates<sup>57</sup>. The site and mechanism of oxidation of nitroglycerin to carbon dioxide has also been investigated<sup>58</sup>. *In vitro* experiments demonstrated that homogenates of the liver, kidney, brain and skeletal muscle oxidized glycerol, a metabolite of nitroglycerin, to CO<sub>2</sub> but could not oxidize nitroglycerin to CO<sub>2</sub>. Evisceration of rats inhibited CO<sub>2</sub> production after the administration of nitroglycerin but not glycerol. Pretreatment of the rodents with phenobarbital or SKF 525A had no effect on nitroglycerin oxidation, nor was there an enhancement of CO<sub>2</sub> release in nitroglycerin-tolerant animals. Thus, CO<sub>2</sub> production may have resulted from biotransformation at extrahepatic sites subsequent to hepatic denitration of nitroglycerin.

### 5.3 Metabolic Fate

Upon oral administration of 10 mg/kg of 1,3-C<sup>14</sup> nitroglycerin to rats<sup>59</sup> 20% of the labeled dose was expired as carbon dioxide with an equal amount of the radioactivity excreted in the urine at the end of 4 hours. TLC-radio-

chromatographic analysis revealed that the cumulative urinary excretion consisted of 7% glycerol, 1% glyceryl-1,2-dinitrate, 0.5% glyceryl-1,3-dinitrate, 4% glyceryl mononitrates and 8% of unidentified water soluble metabolites. Needleman *et al* <sup>58</sup> administered a smaller dose (5 mg/kg) of radioactive nitroglycerin subcutaneously to rats. They observed that 17% of the dose was eliminated as expired CO<sub>2</sub>, with urinary excretion accounting for another 50% of the radioactivity in 0-24 hours. The major urinary metabolites were the glyceryl mononitrates (32% of dose). The sum of the mononitrates and water soluble metabolites (unidentified) accounted for 80% of the excreted label. A small fraction of the labelled dose (1.3%) was excreted as unchanged nitroglycerin.

In a more recent study, Hodgson and Lee<sup>60</sup> administered a very high oral dose, 180 mg/kg (LD 10%), of nitroglycerin to rats. Radioactive CO<sub>2</sub> accounted for 26% of the dose and 40% of the label was eliminated in the urine within 24 hours. These authors showed (Table II) that the major urinary metabolites are glyceryl dinitrate glucuronide (14% of dose), glyceryl mononitrate (11%) and glycerol (7%). This study was the first which showed that conjugation plays a major role in the metabolism of nitroglycerin.

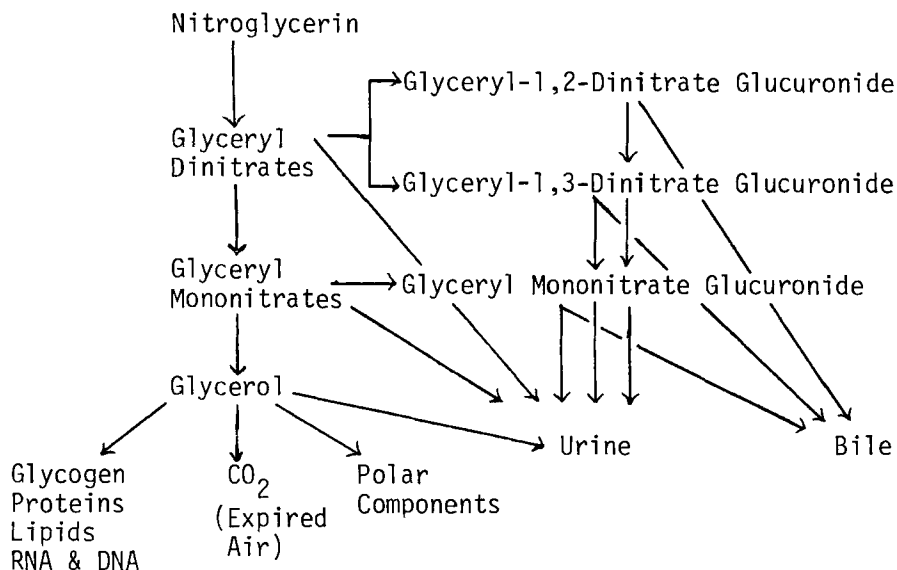
TABLE II<sup>60</sup>

Metabolites of Nitroglycerin in Rat Urine 24 Hours  
After Oral Administration of  
[<sup>14</sup>C] Nitroglycerin (180 mg/kg)

<u>Metabolite</u>	<u>% of Administered Dose</u>
Nitroglycerin	< 0.1
Glyceryl-1,3-dinitrate	0.4 + 0.2 <sup>a</sup>
Glyceryl-1,2-dinitrate	0.7 + 0.4
Glyceryl mononitrate	10.6 + 1.3
Glyceryl-1,3-dinitrate glucuronide	3.5 + 0.4
Glyceryl-1,2-dinitrate glucuronide	10.0 + 0.7
Glyceryl mononitrate glucuronide	1.5 + 0.2
Glycerol	6.9 + 0.8
Unidentified	≈ 6

<sup>a</sup> Mean ± SE of three rats

The metabolic fate of nitroglycerin in the rat can, therefore, be schematically summarized as follows (Fig. 4):



### Fig. 4 Metabolic Fate of Nitroglycerin

The metabolic fate of nitroglycerin in man has not been studied in great detail. So far, only glyceryl mononitrates have been identified as the major urinary metabolite of nitroglycerin in man<sup>61</sup>.

## 6. Pharmacokinetics

### 6.1 Tissue Distribution

Nitroglycerin is rapidly and extensively distributed in the body. Following intravenous administration of radio-labeled nitroglycerin in the rat, Needleman and co-workers<sup>57</sup> found that the apparent distribution phase of unchanged nitroglycerin from blood has a half-life of less than 20 seconds. Tissue radioactivity was not, however, measured in the study. DiCarlo et al.<sup>59</sup>, studied the distribution of {C<sup>14</sup>} after oral administration (10 mg/kg) of {C<sup>14</sup>} nitroglycerin in the same species. They measured the dioxane extractable and non-extractable radioactivity in the tissues as a function of time. The liver and carcass appeared to be the major sites of distribution of absorbed radioactivity. The heart, lung, kidney and spleen took up only small quantities of the radio-label. Significant accumulation of non-extractable radioactivity was shown in the carcass, liver and GI tract, suggesting that nitroglycerin and/or its biotransformation products might be incorporated into the tissues.

This observation agrees with that of a later study<sup>62</sup> which showed that the radioactivity from  $\{^{14}\text{C}\}$  nitroglycerin could be incorporated into rat liver glycogen, lipid, protein, RNA and DNA. Hodgson and Lee<sup>60</sup> also studied the distribution of  $\{^{14}\text{C}\}$  in the rat after oral administration of 180 mg/kg of radioactive nitroglycerin in peanut oil. They found however, no accumulation of absorbed radioactivity in the carcass after 4 hours post dosing. At the same time, about 60% of the radioactivity was detected in the GI tract.

### 6.2 Intravenous Administration

The pharmacokinetics of nitroglycerin are characterized by an extremely rapid plasma clearance of drug. Following intravenous administration in rats (0.35-2.5 mg/kg), plasma nitroglycerin clearance is about 0.6 L/min/kg<sup>63</sup>. In man, plasma nitroglycerin clearance has been reported as 23.6 and 28 L/min following intravenous infusion<sup>64</sup> and sublingual administration, respectively<sup>65</sup>. Since these values are in excess of liver blood flow, it has been suggested<sup>65</sup> that there must be substantial extra-hepatic elimination.

Plasma drug clearance is a function of the apparent volume of distribution and the elimination rate constant, both of which are quite high for nitroglycerin. An apparent volume of distribution of about 3 L/kg has been calculated for nitroglycerin in rats<sup>66</sup>, which is consistent with the extensive tissue distribution discussed earlier. Following doses of 0.7 mg/kg in rats<sup>66</sup> and therapeutic doses in man<sup>64,65</sup>, plasma elimination appears monoexponential with an elimination half-life of approximately 3-4 minutes. Administration of higher doses (2.5 and 3.5 mg/kg) in rats resulted in an apparent biexponential decay with a  $t_{1/2\beta} \approx 15$  min.<sup>63</sup>. It is possible that at therapeutic doses, multi-exponential disposition cannot be characterized because of analytical uncertainties encountered with the extremely low plasma concentrations (< 1 ng/ml) found.

### 6.3 Oral and Topical Administration

The rationale for oral use of organic nitrates has been a controversial subject. Following oral administration and portal vein infusion of nitroglycerin to rats, in doses up to 0.5 mg/kg, Needleman *et al.*<sup>57</sup> observed no blood pressure response and negligible blood concentrations of intact drug. These authors also observed human liver biopsy samples to have metabolic capacity for organic nitrates similar to that found in rats, and they concluded that the systemic availability of nitroglycerin following oral administration is negligible. Clinical studies demonstrating efficacy of orally administered nitroglycerin<sup>67</sup> imply,

however, that systemic availability of nitroglycerin may be significant in man, since the metabolites of nitroglycerin are considerably less active.

The use of topical nitroglycerin has been shown to give sustained hemodynamic effects in man<sup>68</sup>. Following topical administration of a 2% nitroglycerin ointment, equivalent to 16 mg of nitroglycerin, plasma concentrations were similar to those seen after oral administration of a 6.5 mg sustained release capsule<sup>69</sup>. Site dependency in the percutaneous absorption of nitroglycerin has been observed in man<sup>70</sup> and in rats<sup>8</sup> but not in the rhesus monkey<sup>71</sup>. The surface area of application has also been shown to be an important factor in topical absorption when assessed by hemodynamic effects<sup>72</sup>.

## 7. Methods of Analysis

### 7.1 Official Methods

The "Official Methods of Analysis" published by the Association of Official Analytical Chemists<sup>73</sup>, describes two methods for the determination of nitroglycerin. The first involves ether extraction followed by the reduction of nitrogen to ammonia and subsequent determination by titration with acid. A second method utilizes the infrared absorption peak near 7.89  $\mu\text{m}$  and requires a nitroglycerin reference standard for quantitation.

The assay for nitroglycerin developed by Hohman and Levine<sup>74</sup> is the basis for the official USP<sup>75</sup> procedure. This technique uses column chromatography to separate nitroglycerin from its degradation products followed by acid hydrolysis to nitrate ion and subsequent spectrophotometric determination of nitrated phenoldisulfonic acid. Potassium nitrate is used as a reference standard. Both the AOAC reduction method and the USP procedure are useful as primary standardizing procedures for up to milligram quantities of nitroglycerin.

### 7.2 Spectrophotometric

Quantitation of nitrate and nitrite ion following hydrolysis of organic nitrates is possible using colorimetric methods. Spectrophotometric measurement of nitroxyleneol formed from the reaction of hydrolyzed organic nitrate with either 2,4-xyleneol or 2,6-xyleneol is the basis of the xyleneol procedure<sup>76,77</sup>. Application of the Griess reaction and various modifications have been used in biological work for nitrate measurement<sup>78-80</sup>. However, these methods do not possess the requisite sensitivity for the analysis of nitroglycerin in biological fluids during drug therapy.



Several spectrophotometric methods are available for quality control determinations of nitroglycerin in dosage forms. In the assay described by Bell<sup>81,82</sup>, nitroglycerin is hydrolyzed with strontium hydroxide to form nitrite ion. Following diazotization with N-(1-naphthyl) ethylene diamine dihydrochloride, quantitation is achieved by colorimetric determination of the azo dye. The use of strontium hydroxide is said to reduce the interference due to lactose. Since the conversion of nitroglycerin to nitrite is not stoichiometric, absolute quantitation requires a nitroglycerin reference standard. This method has been automated<sup>75,83</sup>. Use of tetramethylammonium hydroxide to hydrolyse nitroglycerin has been reported<sup>84</sup> to produce stoichiometric conversion of 2 moles of nitrite per mole of nitroglycerin as predicted by Hay<sup>85</sup>. It should therefore be possible to use potassium nitrite as a reference standard for the Bell assay with this modification.

A kinetic method has been developed which is suitable for the analysis of single dosage units<sup>15-17</sup>. This assay is based upon the stepwise degradation of nitroglycerin in alkaline alcoholic solutions, with the formation of a chromophoric intermediate. The absorbance maximum at 328 nm was shown to be proportional to the initial nitroglycerin concentration present in the reaction.

The specificity of the USP, Bell and kinetic assays was examined by Morrison and Fung<sup>86</sup>. They found the USP and kinetic assay procedures to be stability-indicating whereas the Bell assay is predictably interfered with by inorganic nitrite. However, under the reaction conditions of the Bell method, the dinitrates, mononitrates and inorganic nitrate did not interfere.

### 7.3 Thin Layer Chromatography

Thin layer chromatography has been used to separate the <sup>14</sup>C-glyceryl nitrates (nitroglycerin and its metabolites) prior to quantitation of the radioactivity. The system reported by Crew and DiCarlo<sup>18</sup> (Table III) is representative of others<sup>23,59</sup> that have been reported.

### 7.4 Polargraphy

The polargraphic behavior of nitroglycerin, pentaerythritol tetranitrate and ethyleneglycol dinitrate has been studied in an ethanol-water system based on the reduction of nitrate at the dropping mercury electrode. Tetramethylammonium chloride was used as the supporting electrolyte. The effects of pH, number of nitrate groups, mercury column height, buffers and solvent on the half-wave potential (measured against the saturated calomel electrode ( $E_{1/2}$  vs. S.C.E.) and the diffusion current (i.d.) was examined<sup>89</sup>.

Using this technique for the assay of single sublingual tablets, Flann<sup>88</sup> reported a  $E_{1/2}$  of -0.91 volts (vs. S.C.E.) and the i.d. to be dependent on nitroglycerin concentration. A non-aqueous polarographic method has also been described by Woodson and Alber<sup>89</sup>.

TABLE III<sup>18</sup>

Thin-Layer Chromatography of Nitroglycerin

TLC plates:	250 $\mu$ silica gel G bound with calcium sulfate	
Solvent:	benzene:ethylacetate:acetic acid (16:4:1)	
$R_f$ values:	nitroglycerin	0.60
	glyceryl-1,3-dinitrate	0.45
	glyceryl-1,2-dinitrate	0.30
	glyceryl-1-mononitrate	0.10
	glyceryl-2-mononitrate	0.10
	glycerol	0.00

### 7.5 Gas Chromatography

Several GC procedures have been described for the analysis of organic nitrates. This technique is especially suitable for determination of nitroglycerin in biological fluids after drug administration. The use of the electron capture detector gives the necessary sensitivity. Table IV gives chromatographic conditions that have been utilized for nitroglycerin determination.

### 7.6 High Performance Liquid Chromatography

Several HPLC methods have been reported for the assay of nitroglycerin in dosage forms. Two normal phase methods are available<sup>98,99</sup> but data is lacking on their specificity. Table V lists the chromatographic conditions of two procedures shown to be specific for nitroglycerin in the presence of degradation products.

TABLE IV

GC Conditions for Nitroglycerin

<u>Reference</u>	<u>Column</u>	<u>Detector</u>	<u>Temperature (°C)</u> (I = Injection port, C = Column, D = Detector)	<u>Sample</u> <u>Analyzed</u>	<u>Sensi-</u> <u>tivity</u> (ng/ml)
90	3.5% QF - 1 on 60-80 Gas Chrom Q.	ECD	I = 160, C = 120, D = 180	5 ml human plasma	0.5
91	3% SP-2401 on 100-120 Supelcoport.	ECD	I = 160, C = 140, D = 180	0.2 ml rat/ human plasma	0.1
92	3% SE-30 on 50-60 Anakrom AB15.	TCD	C = 130, D = 192	Tablet extract	--
93	0.4% OV-17 on 60-80 glass beads	ECD	I = 150, C = 120, D = 150	2 ml human blood or urine	0.1-2
94	3% SE-30 on 100-120 Gas Chrom Q.	ECD	I = 200, C = 150, D = 175	3 ml human blood	?
65	10% OV-101 on 100-120 Chromosorb W-HP	ECD	I = 150, C = 130, D = 200	4 ml human plasma	?
95	30% SE-30 on 80-100 Chromosorb W-HP	ECD	I = 150, C = 130, D = 210	5 ml human plasma	~ 0.5
96	3.8% OV-101 on 80-100 Gas Chrom Q; 2.5% OV-210 on Chromosorb W-HP; 1.1% OV-225 on Gas Chrom Q.	FID	I = 70, C = 70-220 @ 60°/min, D = 225	Nitrocellulose propellants	--
97	3% XE-60, 3.5% QF-1 on 60-80 Gas Chrom Q.	FID	I = 160, C = 150, D = 200	solvent mixtures	--
		ECD	I = 160, C = 120, D = 200		

TABLE V

HPLC Conditions for Assay of Nitroglycerin

	<u>Ref<sup>100</sup></u>	<u>Ref<sup>101</sup></u>
Column	C <sub>18</sub> microparticulate	Alkyl phenyl bonded to silica gel
Mobile Phase	60% MeOH	Acetonitrile-Tetrahydrofuran-Water (26:64:10)
Flow rate (ml/min)	2.0	2.0
Detection	u.v 200 nm	u.v 218 nm
Detection limit	30 ng on column	50 ng on column
Retention time	4 min	10 min

Acknowledgement

Supported in part by NIH grant 22273. We thank Dr. Dinesh Gala for running the infrared and nmr spectra.

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Literature reviewed up to 4/1/80.



# TRIFLUOPERAZINE HYDROCHLORIDE

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## 1. Description

### 1.1 Nomenclature

#### 1.11 Chemical Names

Several chemical names have been used to denote trifluoperazine hydrochloride:

- (a) 10H-Phenothiazine, 10-[3-(4-methyl-1-piperazinyl)propyl]-2-(trifluoromethyl)-dihydrochloride<sup>1</sup>
- (b) 10-[3-(4-methyl-1-piperazinyl)propyl]-2-(trifluoromethyl)phenothiazine dihydrochloride<sup>1</sup>
- (c) Dihydrochloride of 10-[3-(4-methylpiperazine-1-yl)propyl]-2-trifluoromethylphenothiazine<sup>2</sup>
- (d) 2-Trifluoromethyl-10-[3-(1-methyl-4-piperazinyl)propyl]phenothiazine<sup>3</sup>

#### 1.12 Trade Names

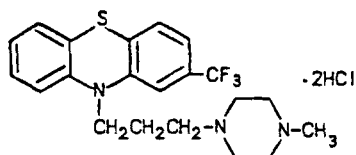
Iatroneural, Jatroneural, Eskazinyl, Eskazine, Stelazine<sup>®</sup><sup>4</sup>, Terfluzine

### 1.2 Formula, Molecular Weight, Structure

#### 1.21 Empirical Formula, Molecular Weight

$C_{21}H_{24}F_3N_3S \cdot 2HCl$                       480.420

#### 1.22 Structure



### 1.3 Appearance, Color, Odor

Both the National Formulary<sup>1</sup> and the British Pharmacopoeia<sup>2</sup> describe Trifluoperazine Hydrochloride as follows:

A white to off-white (cream colored) crystalline powder with little or no odor.

## 2. Physical Properties

### 2.1 Spectral Properties

#### 2.11 Infrared Spectra

Figure 1 is the infrared spectrum of trifluoperazine free base and Figure 2 is the infrared spectrum of the hydrochloride salt of trifluoperazine taken in mineral oil dispersion from 4000-625  $\text{cm}^{-1}$  on a Perkin-Elmer Model 457A. The significant bands in the spectra are assigned as follows:

Free Base		HCl Salt	
Wavelength $\text{cm}^{-1}$	Assignment	Wavelength $\text{cm}^{-1}$	Assignment
1600, 1575, 1500	C=C, aromatic	2700-2100	NH <sup>+</sup>
1330, 1250, 1130	CF <sub>3</sub>	1600, 1570, 1470	C=C, aromatic
830	1,2,4-trisubstituted aromatic	1320, 1340, 1115	CF <sub>3</sub>
750	1,2-substituted aromatic	829	1,2,4-trisubstituted aromatic
		760	1,2-substituted aromatic

The identification and differentiation of phenothiazine type tranquilizers by the IR spectra of salts as derivatives has been reported.<sup>5,6</sup>

#### 2.12 Ultraviolet Spectrum

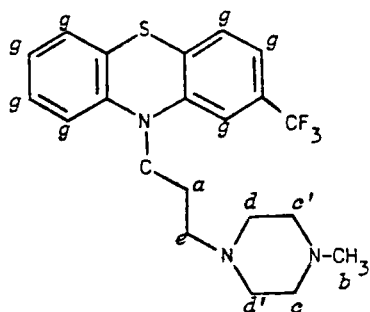
The ultraviolet absorption spectrum of trifluoperazine in 95% ethanol is shown in Figure 3. Maxima at 258 nm ( $\log \epsilon$  4.50) and 307.5 nm ( $\log \epsilon$  3.50) are bands characteristic of a 2-substituted phenothiazine<sup>13</sup>.

The ultraviolet spectrum of trifluoperazine has been used in the analysis of biological specimens<sup>7,9,10,12</sup> as well as in the analysis of the drug itself and its derivatives.<sup>8</sup> The importance of careful control of instrumental parameters such as slit width and the absorption of UV and visible light by phenothiazines has also been reported.<sup>11</sup>

#### 2.13 Nuclear Magnetic Resonance Spectra

##### 2.131 Proton Spectrum

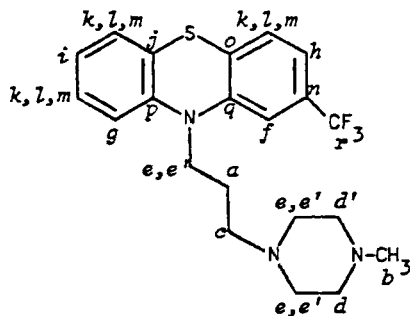
The proton NMR spectrum (Figure 4) was obtained on a deuteriochloroform solution containing approximately 100 mg/ml of trifluoperazine and tetramethylsilane as internal reference standard. The spectrum was obtained on a Perkin-Elmer R32 NMR. The NMR signals are assigned as follows:



<u>Protons</u>	<u>Chemical Shift, ppm</u>	<u>Multiplicity</u>
a	1.94	multiplet
b	2.25	singlet
c, c'	2.3 - 2.6	broad multiplet, signals overlapping
d, d'		
e		
f	3.96	triplet
g (all aromatics)	6.75 - 7.30	broad multiplet, signals overlapping

## 2.132 <sup>13</sup>C Spectrum

The <sup>13</sup>C NMR Spectrum (Figure 5) was obtained on a deuteriochloroform solution of trifluoperazine with tetramethylsilane as a reference. The spectrum was obtained on a Varian Associates Model FT-80 spectrometer. The NMR signals are assigned as follows:



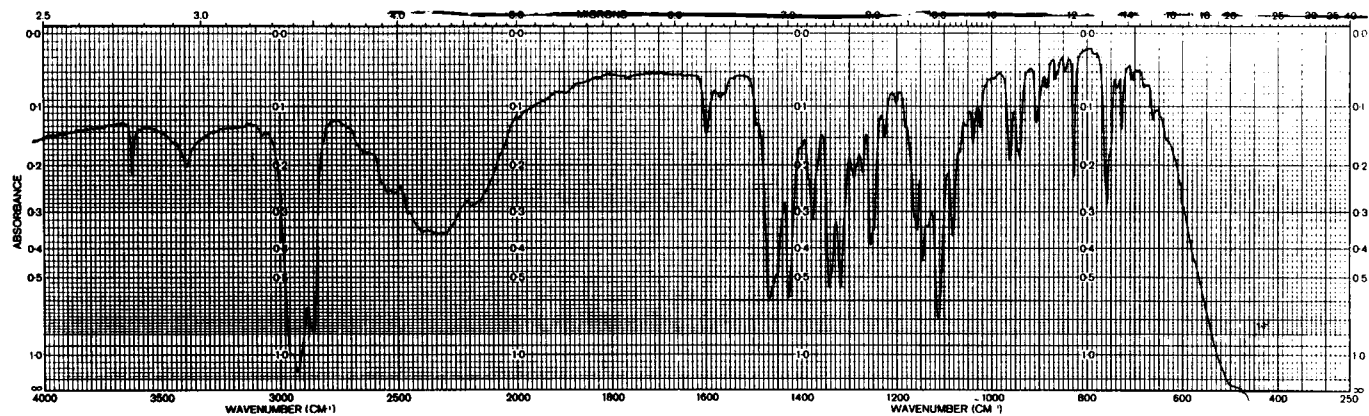


Figure 1: Infrared Spectrum of Trifluoperazine Free Base

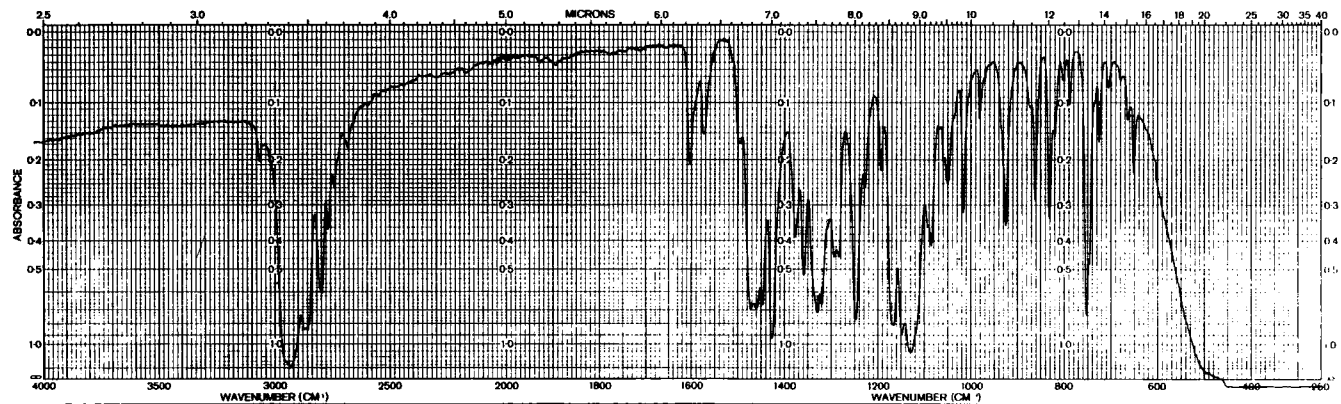


Figure 2- Infrared Spectrum of the Hydrochloride Salt of Trifluoperazine

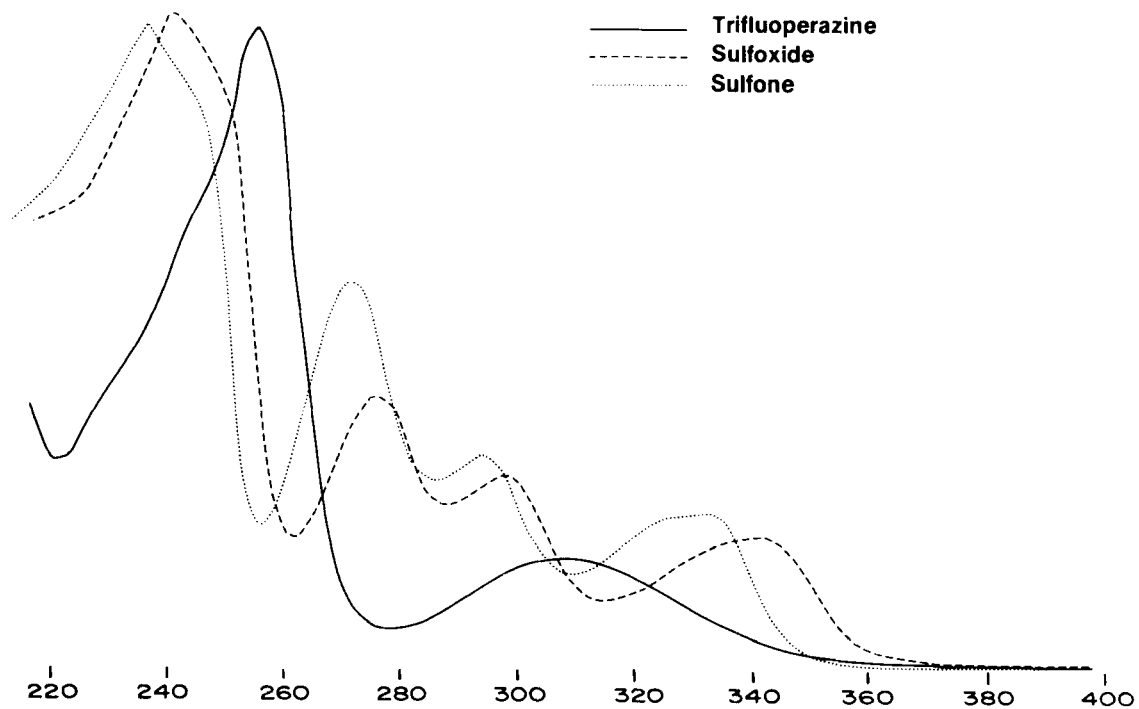
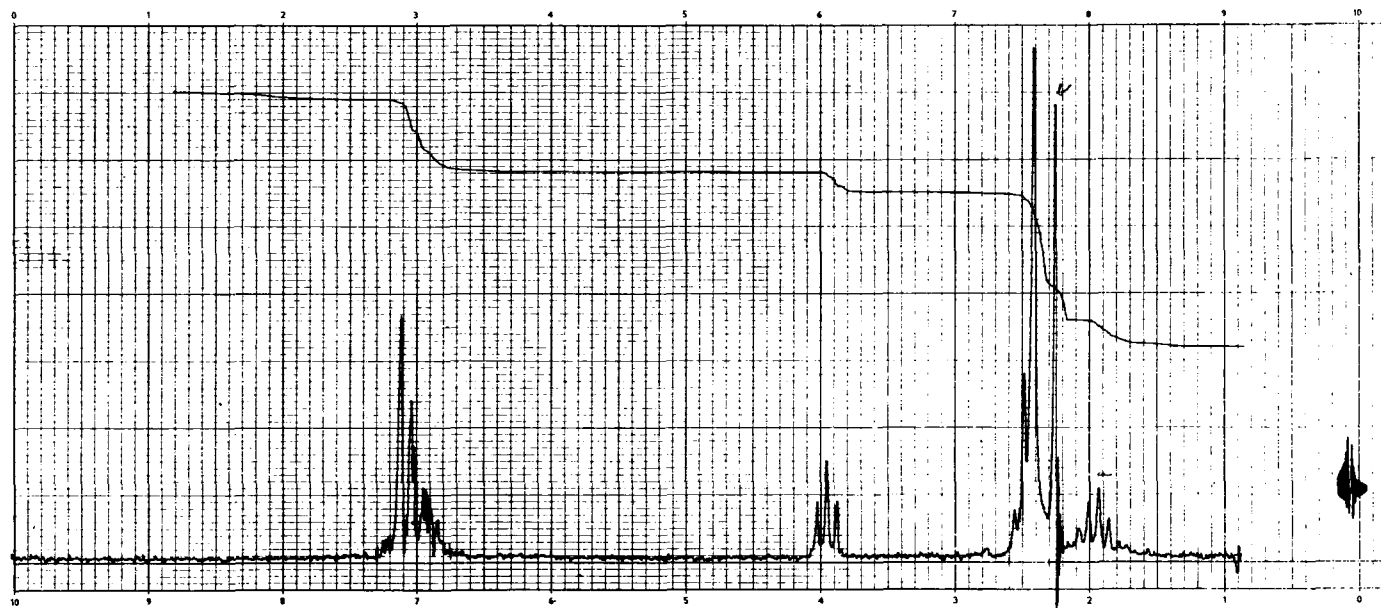


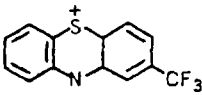
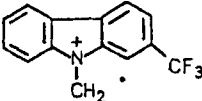
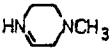
Figure 3- Ultraviolet Absorption Spectrum of Trifluoperazine in 95% Ethanol



*Figure 4- Proton NMR Spectrum*



Table 1 (cont'd)

<u>m/e</u>	<u>Ion</u>
266	
248	
141	$\text{CH}_2\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_3$
127	$\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_3$
113	$\text{CH}_2=\text{N}^+\text{CH}_3$
99	
70	$\text{CH}_2=\text{CHN}^+=\text{CH}_2$ $\text{CH}_3$
36	$(\text{HCl})^+$

Analysis and identification of drugs by mass spectroscopy has been reported.<sup>14</sup>

## 2.2 X-Ray Diffraction Pattern

The X-Ray diffraction pattern of trifluoperazine dihydrochloride is presented in Table 2.

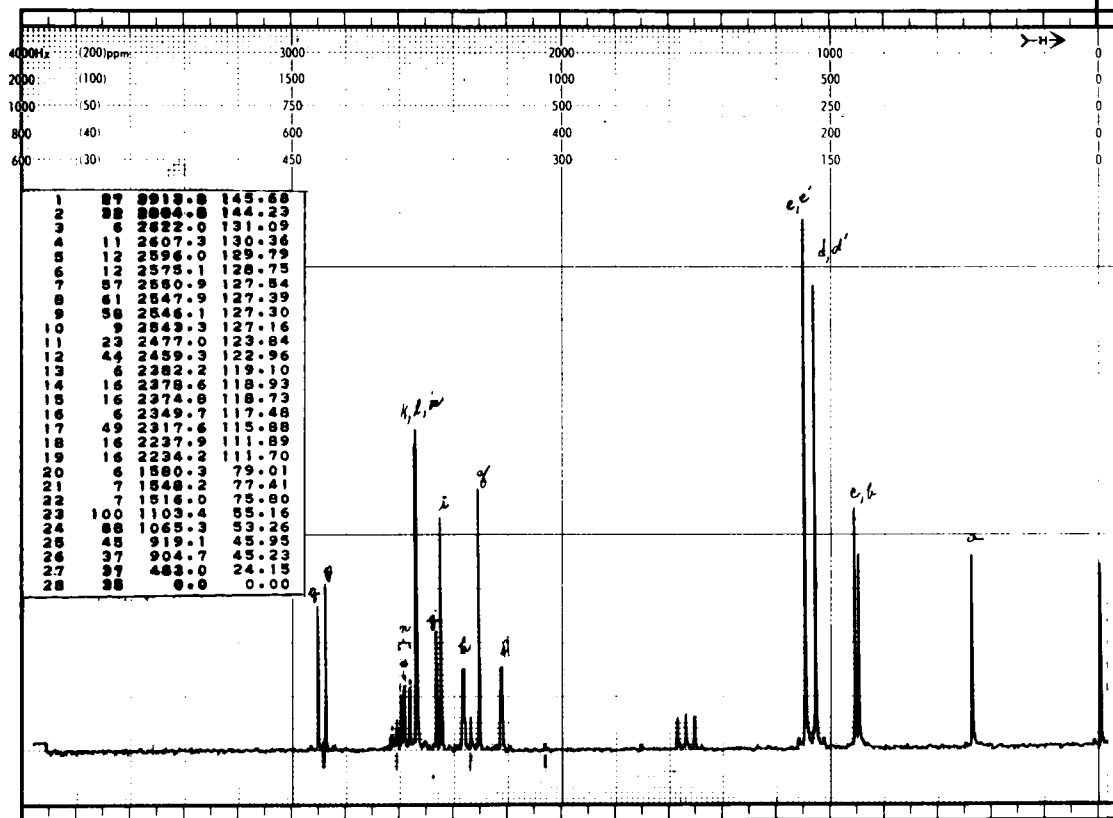


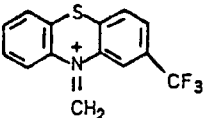
Figure 5-  $^{13}\text{C}$  NMR Spectrum

<u>Carbon</u>	<u>Chemical Shift, ppm</u>
<i>a</i>	24.15
<i>b</i>	45.23
<i>c</i>	45.95
<i>d, d'</i>	53.26
<i>e, e'</i>	55.16
<i>f</i>	111.80
<i>g</i>	115.88
<i>h</i>	118.83
<i>i</i>	122.96
<i>j</i>	123.84
<i>k</i>	127.54
<i>l</i>	127.39
<i>m</i>	127.30
<i>n</i>	129.56 (doublet center)
<i>o</i>	129.79
<i>p</i>	144.23
<i>q</i>	145.68
<i>r</i>	124.29 (quartet center)

#### 2.14 Mass Spectrum

The mass spectrum of trifluoperazine was obtained by direct insertion into an Hitachi Perkin-Elmer RMU-6E low resolution mass spectrometer. The results are presented in tabular form in Table I and as a bar graph in Figure 6

Table I

<u>m/e</u>	<u>Ion</u>
407	$M^+$
392	$(M - CH_3)^+$
388	$(M - F)^+$
363	$(M - C_2H_6N)^+$
350	$(M - C_3H_7N)^+$
307	$(M - HN \text{ (piperazine ring) } N-CH_3)^+$
294	$(M - CH_2N \text{ (piperazine ring) } NCH_3)^+$
280	

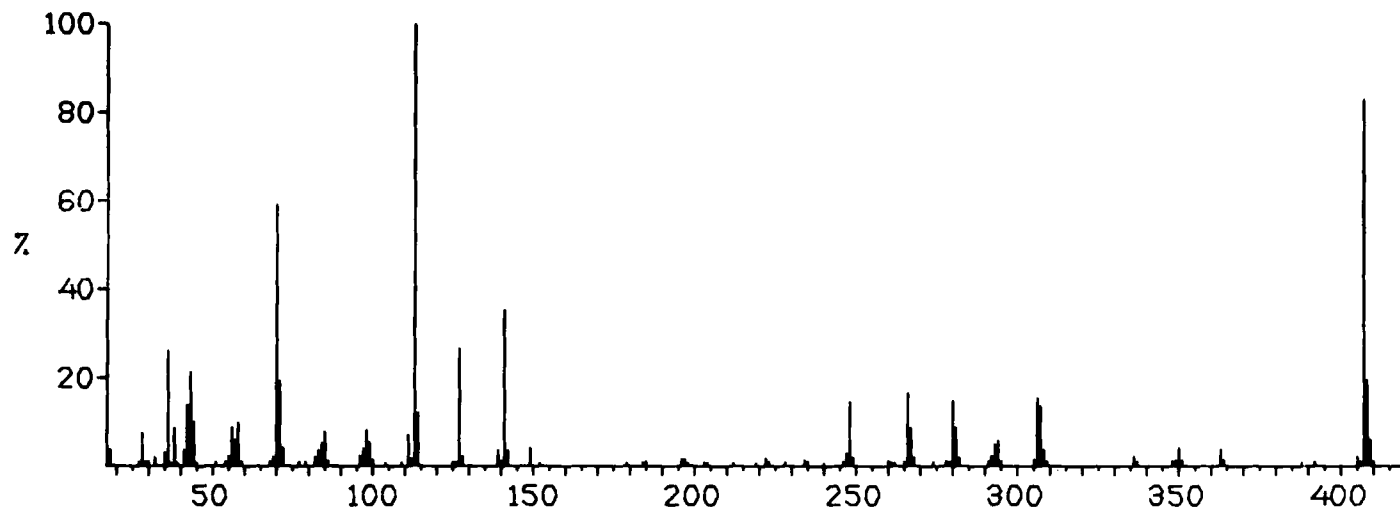


Figure 6 - Mass Spectrum of Trifluoperazine

Table 2X-Ray Diffraction Pattern of Trifluoperazine Dihydrochloride

<u>2 <math>\theta</math></u>	<u>I/I<sub>0</sub></u>	<u>d(A<sup>o</sup>)</u>
10.10	33	8.75
13.80	12	6.44
15.30	98	5.79
15.60	58	5.68
17.30	8	5.12
20.50	100	4.33
21.90	8	4.06
23.20	24	3.83
23.50	24	3.78
23.80	8	3.74
27.60	12	3.23
27.80	16	3.21
30.30	16	2.95
32.50	12	2.75

d = Interplanar spacing (distance).

I/I<sub>0</sub> = Relative intensity based on highest intensity of 100.

2.3 Solubility

<u>Solvent</u>	<u>Grams/100 ml</u>	<u>Reference</u>
water	66	15
water	59	2
0.2N HCl	>66	15
0.2N NaOH	insoluble	15
pH 7.4 buffer	0.0014	16
ethanol (95%)	14.5	15
ethyl ether	insoluble	1
chloroform	1.9	15
benzene	insoluble	1
water	0.0013	17

(a) free base

## 2.4 Apparent Partition Coefficients (K)

In an attempt to correlate structure-activity relationships, several investigators determined the apparent partition coefficients in a variety of hydrophobic-hydrophilic systems. Results of these studies are listed in the following table.

<u>Organic/Aqueous Phase</u>	<u>K</u>	<u>Reference</u>
dodecane/pH 7.0 buffer (30°C)	97	18
chloroform/water (pH 1.0)	0.37	19
chloroform/pH 7.6 buffer	>5000	19
chloroform/0.1N HCl	0.7	20
n-octanol/pH 7 buffer	>10 <sup>5</sup>	21
n-octanol/0.125M KCl	49.3	18

## 2.5 Apparent pKa

The apparent pKa<sub>1</sub> and pKa<sub>2</sub> have been determined using titrimetric and solubility<sup>16</sup> measurements. As reported by many of these investigators, the determination of the pKa of phenothiazines, in general, are difficult to obtain because of their poor water solubility. Thus the use of the term apparent pKa. However, Green<sup>16</sup>, using solubility measurements, did indeed confirm that apparent pKa<sub>2</sub> for trifluoperazine is approximately 8.1 - confirming the results obtained by titrimetric measurements.

<u>Apparent pKa</u>			
<u>pKa<sub>1</sub></u>	<u>pKa<sub>2</sub></u>	<u>*Procedure</u>	<u>Reference</u>
3.9	8.4	titrimetric	22
3.9	8.1	titrimetric	18
4.10	8.36	titrimetric	23
	8.1	solubility	16
	8.3	TLC (a)	24

(a) Thin layer chromatography

## 2.6 Thermal Properties

### 2.61 Melting Range

Trifluoperazine dihydrochloride melts at about 242°C with decomposition<sup>1</sup> using the capillary melting tube method. A melting range of 243-244° was reported by Anderson, et al.<sup>25</sup>

### 2.62 Differential Scanning Calorimetry

The DSC thermogram for trifluoperazine dihydrochloride is shown in Figure 7. It is evident that melting does appear to start at approximately 180°C with subsequent rapid decomposition at about 250°C.<sup>26</sup> The thermogram was obtained at a heating rate of 20°C per minute.

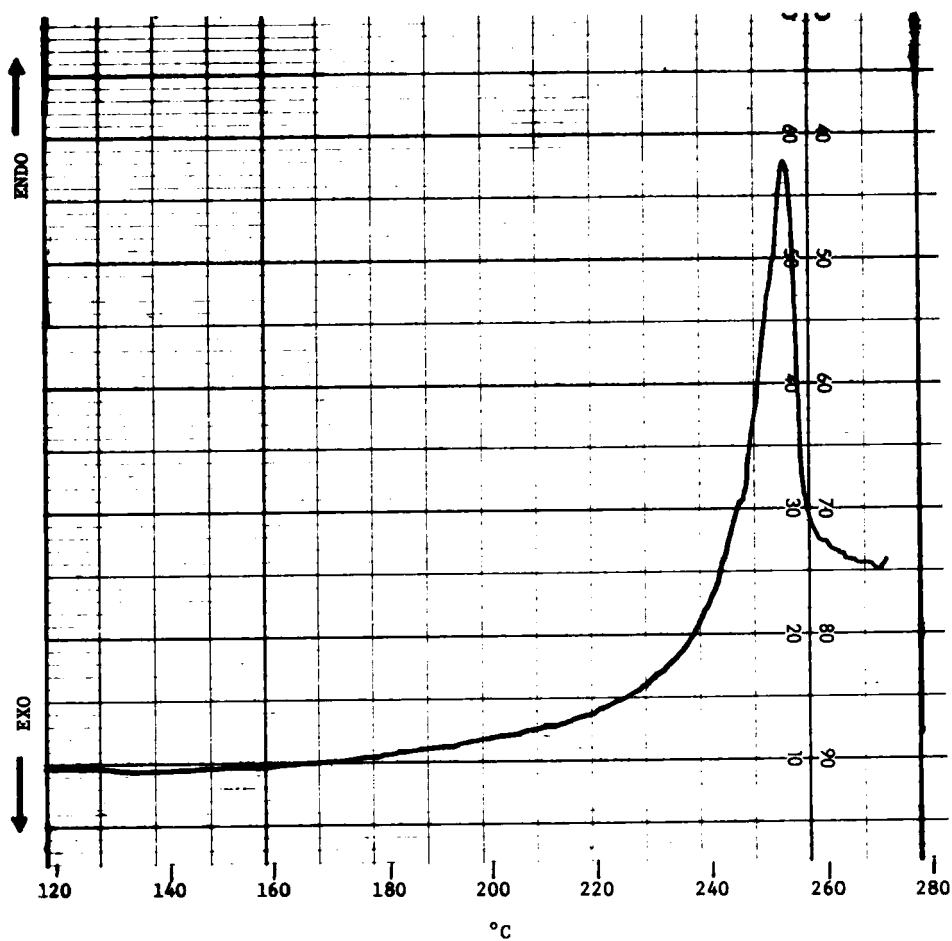
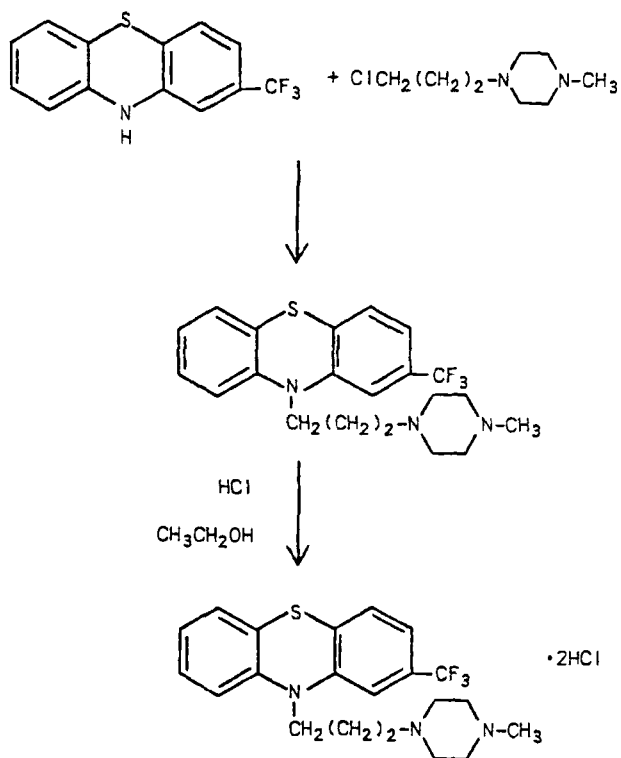


Figure 7 - Differential Scanning Calorimetry Curve of Trifluoperazine Hydrochloride (USP)

### 3. Synthesis

The detailed synthesis of trifluoperazine dihydrochloride is described by Craig, *et al*<sup>27</sup> and Anderson, *et al*<sup>25</sup>. The schematic is illustrated below.





#### 4. Identification

##### 4.1 Derivatives

Several salts have been prepared that can be used for identification purposes (Table 3). As the preparation of the sulfoxide of trifluoperazine can be easily prepared, it has also been used for the identification of the parent compound. (refer to Figure 3)

Table 3  
Salts of Trifluoperazine

<u>Salt</u>	<u>Melting Range</u>	<u>Reference</u>
Dihydrochloride	$\sim 242^{\circ}(a)$	1
Picrate	242.0 (decomp)	6
Reineckate	183.5 - 186.0 $^{\circ}$ (decomp)	6
Dimalate	193 - 194 $^{\circ}$	39
	196 - 197 $^{\circ}$	38
Dimethylsulfonate	257 - 258 $^{\circ}$	38
Difumarate	215 $^{\circ}$	38
Disuccinate	130 - 132 $^{\circ}$	38
Pamoate	152 - 167 $^{\circ}$	38
Benzophenonedicarboxylate	158 - 161 $^{\circ}$	38
Dipyromellitate	>240 $^{\circ}$	38
Dimethiodide Salt	162 - 163 $^{\circ}$	40
Trifluoperazine (free base)	$\sim 80^{\circ}$	38
Trifluoperazine Sulfoxide Dihydrochloride	173 - 175 $^{\circ}(b)$	27

(a) Refer to Section 2.6

(b) With 3 mols H<sub>2</sub>O

##### 4.2 Color Tests

Reactions with color reagents has been the method of choice for differentiating trifluoperazine, its degradation products, and metabolites after a preliminary separation by thin layer and paper chromatography.<sup>29,31-33, 36,37</sup> A listing of several of these color reagents are given in Table 4.

Table 4Identification of Trifluoperazine with Color Reagents

<u>Reagent</u>	<u>Response</u>	<u>Reference</u>
Bromine water + H <sub>2</sub> SO <sub>4</sub>	cherry-red	28
Selenious Acid	amber brown+green	28
Concentrated HNO <sub>3</sub>	pink+yellow	6,28
68% HNO <sub>3</sub>	purple+yellow	29
1% Cobalt acetate + isopropylamine	light blue	6
10% aq. Chloramine-7	creamy blue	6
Palladium chloride	orange brown	6
Uranium nitrate	orange	6
Ammonium vanadate	yellow brown	6
Silver nitrate	creamy white	6
Keller Test (FeCl <sub>3</sub> )	pink+orange	29
2% aq. FeCl <sub>3</sub>	red+violet	31
Ceric sulfate	red+colorless	30
40-50% H <sub>2</sub> SO <sub>4</sub>	orange	31, 33, 34
Folin-Ciocalteu	cameo	32
FPN Reagent	pink	32
Mandelin	flesh	32
Cinnamylaldehyde	flesh	32
Furfural	cameo	32
FeCl <sub>3</sub>	amber	1

4.3 Microscopy

Andres<sup>33,42</sup> was successful in using microcrystalline reactions to differentiate between phenothiazine type tranquilizers. Table 5 contains the results for trifluoperazine only.

Table 5

<u>Reagent</u>	<u>Description of Crystals</u>	<u>Sensitivity of Detection (µg/µl)</u>
Ammonium reineckate	amorphous	---
Picric acid	yellow, birefringent rosettes	0.1
Stannous Chloride	colorless, weakly birefringent irregular rosettes and long needles	0.1
Platinum bromide	amorphous	---
Gold chloride	reddish-brown, weakly birefringent needles, dense rosettes	0.1

In a subsequent collaborative study<sup>6</sup>, Andres recommended that stannous chloride was the reagent of choice to identify trifluoperazine. Fulton<sup>43</sup>, in an extensive study of phenothiazines, was able to characterize and distinguish trifluoperazine from other phenothiazines via the color of crystals formed with gold, platinum, and palladium reagents (Table 6).

Table 6

Color of Trifluoperazine in Microcrystal Tests

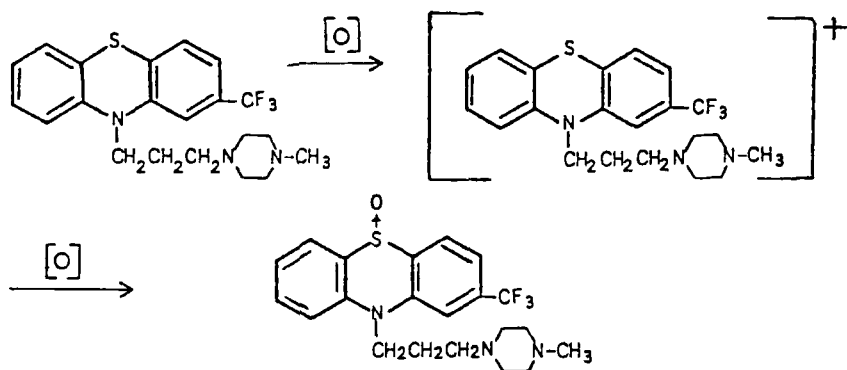
<u>Reagent</u>	<u>Color Obtained</u>
HAuCl <sub>4</sub> in (1+1)H <sub>2</sub> SO <sub>4</sub>	orange
H <sub>2</sub> PtCl <sub>6</sub> aq(10), to (2+1) acetic acid solution, to dryness	purple and violet
H <sub>2</sub> PdCl <sub>4</sub> in (1+35)H <sub>2</sub> SO <sub>4</sub> , to (2+1) acetic acid solution; then evaporated	light salmon deep purple
H <sub>2</sub> PtCl <sub>6</sub> in diluted HClO <sub>4</sub> -acetic acid	brigh sky blue
H <sub>2</sub> PdCl <sub>4</sub> in diluted HClO <sub>4</sub> -acetic acid	red (purple to pale orange)

4.4 Miscellaneous Identification Tests

Ultraviolet and infrared absorption have been used as identity tests<sup>1,34</sup> the R<sub>f</sub> and R<sub>t</sub> of thin layer chromatography and gas liquid and high performance liquid chromatography, respectively, have also been used. These will be cited in subsequent sections. Proton and C-13 nuclear magnetic resonance spectra and mass spectrometry are currently in use. Coupled techniques, i.e. GLC/mass spec, GLC/IR and HPLC/mass spec<sup>38</sup>, are now more commonplace in identifying components from biological tissues.

5. Stability and Degradation

Trifluoperazine is subject to air and light induced oxidative degradation. The mechanism<sup>44,46</sup> can be considered a two-step reaction involving the intermediate formation of a semiquinone free radical which is then oxidized to the sulfoxide.



The formation of the sulfoxide can be readily followed spectrophotometrically. As the oxidation proceeds, the wavelength maximum, 255 nm, for trifluoperazine falls with the concomitant increase in the wavelength maximum for the sulfoxide at 278 nm. Similarly, degradation can be readily followed by many of the methods cited in Section 7.6 and/or quantified by methods listed in Section 7.63-7.65.

Aqueous acid solutions of trifluoperazine, flushed with nitrogen, and kept in the dark, are stable for several days. However, in the light and especially UV light, degradation occurs rapidly. Within 15 minutes and under UV light, discoloration of the solution is evident. In ethanol or acidified ethanol, no such degradation is observed within 48 hours<sup>38</sup>.

Trifluoperazine dihydrochloride stored at room temperature for up to two years did not show degradation<sup>38</sup>.

Lever and Hague<sup>47</sup> observed that on diluting concentrated solutions of trifluoperazine with common diluents used under clinical situations (i.e. cola, coffee, tea, grape and apple juice) there was a color change and turbidity and or precipitation within two hours at room temperature. They recommended dilutions be made freshly and with distilled water only.

## 6. Metabolism

### 6.1 Metabolic Products

The *in vivo* and *in vitro* metabolism of trifluoperazine have been extensively studied by many investigators. The following schematic abstracted from several publications<sup>12,48-54</sup> indicates several pathways to the metabolic products identified. A summary of the findings along with the identification and/or quantitative techniques used to establish the amounts present are listed in Table 7. The cited references contain information regarding the pharmacokinetics of this compound as it relates to the mode of administration, and the amounts of the metabolites present in the various tissues.

### Metabolic Scheme of Trifluoperazine

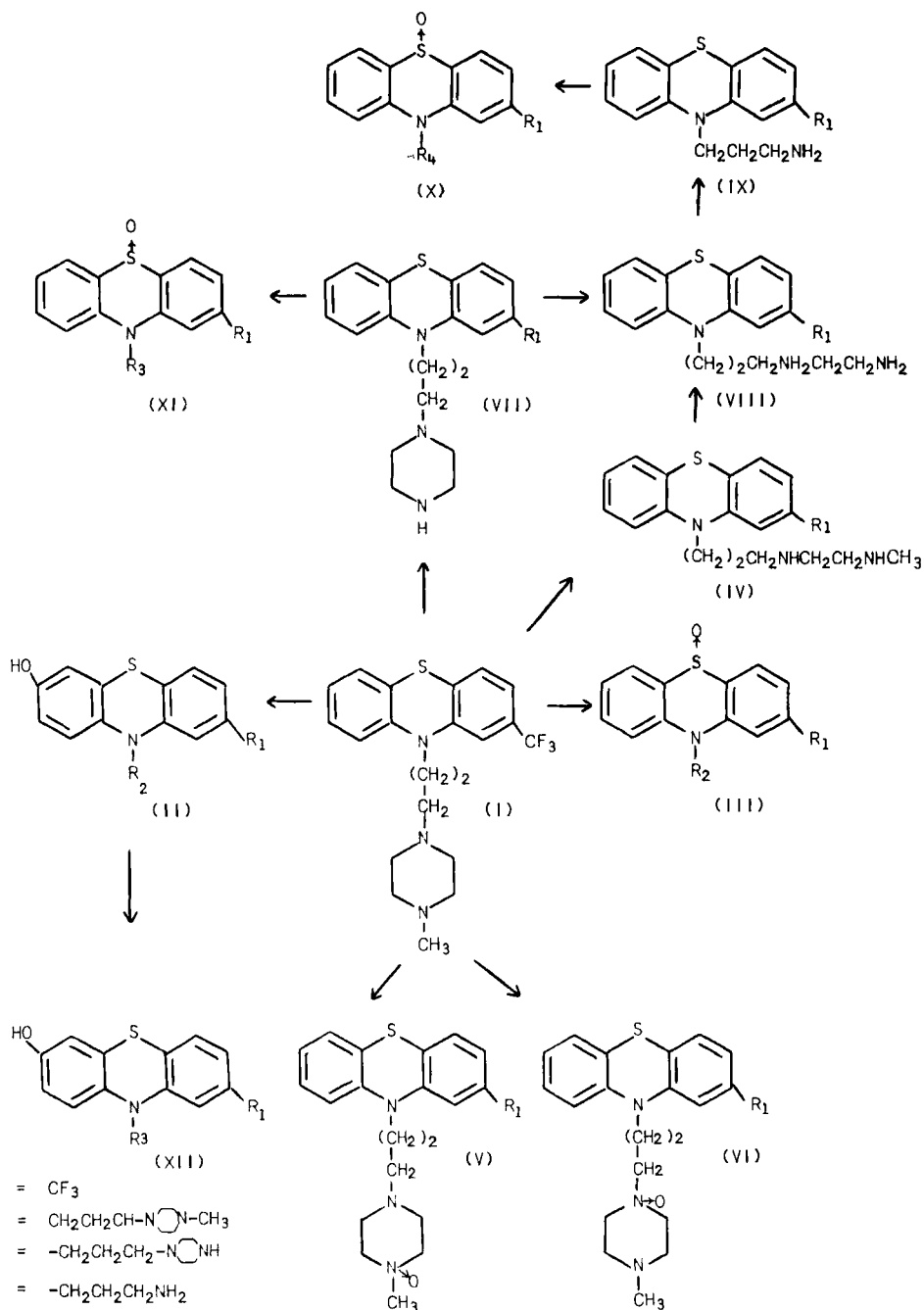


Table 7

<u>Animal</u>	<u>Tissue</u>	<u>Metabolite Identified and/ or Quantified</u>	<u>Analytical Techniques Used</u>	<u>Reference</u>
Rat	Liver Kidney Brain Urine	I, IV, VII, VIII I, IV, VII, VII I, VII X	TLC <sup>a</sup> , MS <sup>b</sup>	48,49
Man	Urine Brain Plasma	I, VII, X I, IV I, IV	TLC, MS SF <sup>e</sup> SF <sup>e</sup>	48,49
Rat	Liver Microsomes	I, III, V, VI, VII, X	GLC <sup>d</sup> , MS	50
Rat	Brain Liver Lungs Kidney	VII I, II, IV, VII, VIII, XII I, III, VII I, III, VII	TLC, UV <sup>c</sup>	51
Rat	Liver Microsomes	I One de-alkylated analogue Two hydroxylated analogues	TLC, UV	52
Rat	Urine	I, II	TLC, UV	53
Rat	Liver Microsomes	VII	TLC	54
Rat	Urine	III, XI	TLC, UV	12

(a) Thin layer chromatography

(b) Mass spectrometry

(c) Ultraviolet absorption

(d) Gas liquid chromatography

(e) Spectrofluorometry

Using  $^{35}\text{S}$  tagged trifluoperazine, Flanagan, et al<sup>55</sup> showed that only about 11-13% of the orally administered dose was detected in the urine of nonfasted rats after 96 hours. Of this amount, 80-85% was found in the 24 hour urine collection. Using fasted rats, only about 9% of the total dose was detected with about 90% found in the 24 hour collection.

Using 24 hour urine collections, West, et al<sup>56</sup> found that trifluoperazine was extensively metabolized by man with unchanged trifluoperazine accounting for less than 1% of the dose; the sulfoxide was 1-6% of the dose; and that the excretion of the trifluoperazine and its metabolite was dose dependent.

## 6.2 Biological Half-Life

Schmalzing and Breyer<sup>57</sup> showed that when trifluoperazine is administered intravenously to male rats, the biological half-life for the trifluoperazine in brain, lung, kidney, and plasma was approximately 2.5 hours, and much longer in the liver. After oral administration, the concentration in the liver was the same as after the intravenous dosage.

## 6.3 Protein Binding

Nambu and Nagar<sup>58</sup> studied the binding of trifluoperazine to bovine serum albumin using an equilibrium dialysis method and a gel filtration procedure. They showed that binding increased with pH, the order of increase was dependent on the ion species with citrate>succinate>phosphate>acetate, was correlated with surface activity, and increased with the partition coefficient in dodecane/water system. Binding to BSA in 1/30 M phosphate at pH 7.00 at 10°C was 82-87%. (The results suggested that a hydrophobic interaction takes part in the binding.) Zia and Price<sup>60</sup> apparently reached the same conclusion when they used 2-(4'-hydroxybenzeneazo)benzoic acid as a spectrophotometric probe and measured the difference absorption spectra with binding of trifluoperazine to bovine serum albumin.

Gabay<sup>61</sup> and Huang<sup>62</sup> studied the binding of trifluoperazine to human serum albumin, as well as that from the dog, rat, rabbit, pig, horse, sheep, goat, and chicken. They also used a UV difference spectrophotometric method and an intrinsic protein fluorescence quenching method. From the shapes of the UV difference spectra, which were essentially identical, indicated that the overall binding site environment (hydrophobic) of the ten species were similar.

## 7. Methods of Analysis

### 7.1 Elemental Analysis

Conventional procedures for the determination of C, H, N, S, Cl, and F yielded the following results on a sample which passed NF XIV specifications.<sup>62</sup>

Element	<u>%</u>	
	<u>Found</u>	<u>Theory</u>
C	52.26	52.50
H	5.31	5.46
N	8.72	8.75
S	6.72	6.67
Cl	14.87	14.76
F	11.87	11.86

## 7.2 Titrimetric Analysis

Several titrimetric procedures have been reported for the assay of trifluoperazine dihydrochloride:

7.21 Titration with perchloric acid in glacial acetic acid is apparently the most frequently used.<sup>63</sup>

The sample is dissolved in glacial acetic acid, mercuric acetate T.S. is added and the titration effected with standardized 0.1N perchloric acid in glacial acetic acid to the blue-green end-point of crystal violet. Each ml of 0.1N perchloric acid is equivalent to 24.02 mg of trifluoperazine dihydrochloride. The end-point can also be determined potentiometrically using glass-calomel electrodes.<sup>64</sup>

7.22 Titrant: Ceric Sulfate  
Detection: Photometric Endpoint

Agarwal and Blake<sup>30</sup> employed a photometric titration procedure. They titrated an acid solution of the sample with 0.02N ceric sulfate, detecting the endpoint photometrically at the wavelength of maximum absorbance of ceric sulfate, 420 nm. An excellent correlation with the method noted in Section 7.21 was obtained.

7.23 Titrant: Ceric Sulfate  
Detection: Colorless Endpoint

The sample, dissolved in dilute sulfuric acid is titrated with ceric sulfate to a colorless endpoint. The equivalence point corresponds to the addition of two moles of ceric ion per mole of trifluoperazine dihydrochloride. The acid-stabilized colored free radical is discharged when the oxidation to the 'sulfoxide' is completed.<sup>65</sup>



### 7.3 Complexometric Analysis

Precipitation of the trifluoperazine as its mono-Reineckate salt with an excess of the precipitating reagent and titrating the excess bromometrically is the basis of the method proposed by Olech.<sup>60</sup> The method is rapid, requiring only milligram amounts of sample and with an error of  $\pm 1.0 - 1.5\%$ . Gajewska<sup>67</sup> used an excess of lead, cadmium, copper, or zinc picrate to precipitate trifluoperazine. The lead picrate forms an insoluble complex, while the others form 5:3 complexes. Titration of the excess cation is made with standardized EDTA.

### 7.4 Spectrophotometric Analysis

Trifluoperazine dihydrochloride can be assayed by ultraviolet spectrophotometry in dilute hydrochloric acid at its maximum wavelength ( $\sim 255$  nm) or via a two point analysis ( $\text{Abs}_{255} \text{ nm} - \text{Abs}_{278} \text{ nm}$ ).<sup>38</sup>

Several approaches to the assaying of trifluoperazine in various commercial preparations have been reported. The British Pharmacopoeia<sup>34</sup> and the National Formulary<sup>63</sup> methods involve an extraction followed by the UV readout of suitably diluted solutions; the former report a method for tablets, the latter for tablets, injection, and syrup.

Alternate procedures were proposed by Watson, et al,<sup>68</sup> for the analysis of trifluoperazine in tablets. In their first procedure, they partitioned a trifluoperazine-bromocresol purple complex between an aqueous pH 6 buffer and benzene-isoamyl alcohol and measured the absorbance of the yellow colored organic phase at 410 nm. In the second method, a 1% hydrochloric acid extract is passed through an alkaline diatomaceous earth column, and the trifluoperazine eluted with chloroform. The chloroform extract is mixed with methanol-hydrochloric acid and the solution measured at 259 nm. These procedures eliminated potential interferences not accommodated by the British Pharmacopoeia procedure.

A differential spectrophotometric method was developed by Davidson<sup>69</sup> which precluded interferences from the photochemical decomposition product (sulfoxide) and excipients including the conventional coloring and flavoring agents. The sample is treated with peroxyacetic acid to rapidly and quantitatively convert the trifluoperazine to its sulfoxide. The difference absorption maximum at 353 nm is a measure of the trifluoperazine. This procedure has been used with sustained-release capsules, as well as other conventional dosage forms.

A highly specific procedure for the phenothiazine nucleus in biological tissues was reported by Wallach and Biggs<sup>7</sup>. A characteristic oxidation product is obtained when the alkaline-extracted phenothiazine is treated with cobalt (III) ion and is stable in the hexane-tertiary butyl alcohol used. The wavelength maximum occurs at 272 nm and the assay is linear over a range of 0.5 - 50.0 mcg/ml.

Huang and Bhansali<sup>53</sup> separated the trifluoperazine and its sulfoxide in urine using thin layer chromatography and after a quantitative elution from the plate determined the amount of each present spectrophotometrically. Using deproteinized human blood and liver (with 5N HCl), followed by extraction of alkalinized solution, Stevens, et al<sup>70</sup> quantified the amount of trifluoperazine spectrophotometrically. Recoveries of 60-76% were obtained.

Using Sephadex LH-20, Malcolm<sup>71</sup> separated the trifluoperazine and then determined the amount present in the various fractions spectrophotometrically to determine the concentration. Reference samples were similarly treated.

### 7.5 Spectrofluorometric Analysis

The fluorescence spectrum of a phenothiazine is unique and thus can be used to quantify this specific compound in biological tissues, solutions and tablets.

Mellinger and Keeler<sup>72</sup> showed that when trifluoperazine is treated with  $\text{KMnO}_4$  in acid, the fluorescence shifts to shorter wavelengths with an increase in intensity and concomitantly, the excitation spectrum changes to form a characteristic wavelength pattern of four distinct peaks. These authors used this procedure for the qualitative identification of phenothiazines showing that 0.6 - 0.8  $\mu\text{g/ml}$  of body fluid could be detected. This was about a five-fold increase over ultraviolet absorption procedures. A subsequent report by these same authors<sup>73</sup> showed that spectrofluorometric analysis could be used to quantitate trifluoperazine in biological tissues, ampuls, and tablets at the final concentration of 2 to 20  $\text{ng/ml}$ .

Ragland and Kenross-Wright<sup>74</sup> found that if the oxidation was effected with hydrogen peroxide in 50% acetic acid, the fluorescence spectrum was more stable and more intense. They subsequently used this procedure to quantify nanogram quantities of the phenothiazine in blood serum, brain tissue, and liver.<sup>75</sup> Tompsett<sup>76</sup> confirmed the applicability and reliability of this method for the quantitation of trifluoperazine in blood serum.

West, *et al*<sup>56</sup> used spectrofluorometry to determine both trifluoperazine and its sulfoxide in urine, plasma, and brain. Recoveries of 68-80% were obtained on 10  $\mu\text{g/ml}$  of urine solutions. Their results were in excellent agreement of those obtained by Spano, *et al*<sup>77</sup> who used a specific radioisotope procedure.

### 7.6 Chromatographic Methods of Separation

#### 7.61 Paper Chromatography

Chromatography on paper and modified papers using an assortment of mobile phases has been used to separate trifluoperazine and its metabolites. Several of the mobile phases and stationary phases are listed in Table 8 and detection methods in Table 9. Paper chromatography has been used in analyzing biological tissues.<sup>33,81,82</sup>

Table 8

Paper Chromatography of Trifluoperazine

<u>Mobile Phase</u>	<u>Stationary Phase</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
IN Sodium Formate	Whatman 3MM	0.27	33
IN Sodium Formate- n-propanol (90:10)	Whatman 3MM	0.25	33
IN Sodium Formate- IN ammonia (90:10)	Whatman 3MM	0.25	33
IN Sodium Formate- 90% Formic acid (97:3)	Whatman 3MM	0.60	33
IN Sodium Acetate	Whatman 3MM	0.17	33
IN Sodium Acetate- n-propanol (90:10)	Whatman 3MM	0.35	33
Sodium Chloride- n-propanol (92:8)	Whatman 3MM	0.55	33
Butanol-Water-Citric Acid (870:130:4.8 g)	Whatman #1 impregnated with 5% sodium dihydrogen citrate	0.34	36 .
pH 4.58 Acetate Buffer, run at 95°C	Whatman #1 impregnated with 10% tributyrin	0.06,0.09	36,81
pH 7.4 Phosphate Buffer, run at 86°C	Whatman #1 impregnated with 10% tributyrin	0.03	36
n-Butanol-HCl-Water (6:1:7.5)	Whatman #1 impregnated with citric acid-phos- phate buffer, pH 4.0	0.91	78
n-Butanol-Acetic Acid- Water (6:1:7.5)	Whatman #1 impregnated with citric acid-phos- phate buffer, pH 4.0	0.88	78
Isobutyl alcohol-Propionic acid-water (10:1:4.5)	Whatman #1 impregnated with citric acid-phos- phate buffer, pH 4.0	0.91	78
5% Ammonium Sulfate sat- urated with Isobutanol	S&S #576, Whatman #1 or 4	0.23	79
Cyclohexane-Benzene (9:1)	Several papers impreg- nated with formamide- 5% ammonium formate	0.78	80

Table 9  
Spray Reagents for Detection of Trifluoperazine  
(Paper Chromatography)

<u>Reagent</u>	<u>Color</u>	<u>Reference</u>
40% H <sub>2</sub> SO <sub>4</sub>	orange	33
Dragendorff's potassium iodoplatinate	purple	generally used
Modified <sup>a</sup> conc. H <sub>2</sub> SO <sub>4</sub>	red	81
Modified <sup>a</sup> Marquis	red	81
Modified <sup>a</sup> Mandelin	orange→red	81
Modified <sup>a</sup> Fröhde	red	81
Modified <sup>a</sup> Mecke	red→brown	81
Palladium Chloride (1%)	red-orange	81
Bromine water	dark green	81
UV, 263 nm	bluish yellow	33
UV, 254 nm	purple yellow	81

(a) Treated with sodium sulfate to reduce rate of reaction.

#### 7.62 Thin Layer Chromatography

A significant number of mobile phases have been used to chromatograph trifluoperazine on silica gel, modified silica gel, and alumina, and are listed in Table 10 along with the respective R<sub>f</sub>s obtained. Similarly, a large number of detection reagents, including spray reagents, have been used to detect trifluoperazine.

Thin layer chromatographic separations have been used to separate trifluoperazine from its metabolites and all subsequently identified using differential spray reagents (Table 10), spectrophotometric procedures, and mass spectrometry. Further, on isolation of these materials, they were quantified using spectrophotometric procedures. Table 11 lists the tissues studied and the 'read-out' used for the qualitative and/or quantitative analysis.

Table 10TLC Systems

<u>Mobile Phase</u>	<u>Adsorbent</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
Cyclohexane-Benzene-Diethylamine (75:15:10)	Silica Gel-0.1M KOH	0.45	83
Methanol	Silica Gel-0.1M KOH	0.49	83
Acetone	Silica Gel-0.1M KOH	0.19	83
Methanol	Silica Gel-0.1M KHSO <sub>4</sub>	0.10	83
Ethanol (95%)	Silica Gel-0.1M KHSO <sub>4</sub>	0.02	83
Ethylacetate-Methanol-Ammonia (85:10:5)	Silica Gel	0.72	84
Ammonium Acetate-Methanol (10 ml 15%:40)	Silica Gel	0.63	85
Methanol-12N Ammonium Hydroxide (T00:1.5)	Silica Gel	0.57	32
Cyclohexane-Diethylamine-Benzene (75:20:15)	Silica Gel	0.54	32
Acetone	Silica Gel	0.12	32
Chloroform-Methanol (90:10)	Silica Gel	0.52	32
Benzene-Ethanol-12N Ammonium Hydroxide (95:15:5)	Silica Gel	0.56	32
Ethylacetate-Acetone-1:1 Ammonium Hydroxide in Ethanol (90:45:4)	Silica Gel	0.44	86
Ethanol-Water-Acetic Acid (20:20:1)	Silica Gel	0.28	87
Chloroform-Methanol (100:10)	Silica Gel	0.45	88
Ethylacetate-Chloroform-Methanol-0.1M Sodium Acetate pH 4.7 buffer (54:23:18:5)	Silica Gel	0.33	89

Table 10 (continued)

<u>Mobile Phase</u>	<u>Adsorbent</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
Benzene-Dioxane-Ammonia (60:35:5)	Silica Gel	0.69	31
Ethanol-Acetic acid-Water (50:30:20)	Silica Gel	0.33	31
Methanol-Butanol (60:40)	Silica Gel	0.40	31
t-Butylalcohol-1N Ammonia (90:10)	Silica Gel	0.18	33
n-Propanol-1N Ammonia (88:12)	Silica Gel	0.33	33
Ether, saturated with water	Silica Gel	0.09	33
70% Methanol	Silica Gel	0.34	33
85% n-propanol	Silica Gel	0.12	33
n-Butanol saturated with 1N Ammonia	Silica Gel	0.51	33
Isopropanol-Chloroform-1.3N Ammonia water (16:8:1:1)	Silica Gel	0.67	51
Acetone-Isopropanol-1N Ammonia (27:21:12)	Silica Gel	0.77/0.81	12/51
1,2-Dichloroethane- Ethylacetate-Ethanol-Acetic acid-Water(15:26:12:8:7.5)	Silica Gel	0.51/0.32	12/51
Isopropanol-Chloroform-25% Ammonia-Water (32:16:25:1)	Silica Gel	0.83	12
Chloroform-Ethanol-Ammonia (80:20:1)	Silica Gel	0.80	52
Benzene-Dioxane-Diethylamine- Ethanol (50:40:5:5)	Silica Gel	0.85	37
Acetone-Ethyl acetate-Ethanol (5:4:1) saturated with Ammonium lactate pH 3	Silica Gel	0.14	90
Acetone-Ethyl acetate-Ethanol (5:4:1) saturated with Ammonium lactate pH 7	Silica Gel	0.30	90

Table 10 (continued)

<u>Mobile Phase</u>	<u>Adsorbent</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
Acetone-Ethyl acetate-Ethanol (5:4:1) saturated with Ammonium lactate pH 9	Silica Gel	0.17	90
Benzene-Acetone (95:5)	Alumina (neutral)	0.20	91
Benzene-Acetone (95:5)	Alumina (basic)	0.40	91
Benzene-Acetone (90:10)	Alumina (basic)	0.53	91
Water-Acetone (70:30)	Cellulose	0.35	91
Toluene-Chloroform-Methanol- Ammonium Hydroxide (60:40:10:0.2)	Silica Gel	0.44	92
Ethyl acetate-n-Propanol- Ammonia (70:25:4)	Silica Gel	0.53	93
Ethyl acetate-Dichloroethane- Ammonia (80:20:5)	Silica Gel	0.47	93
Cyclohexane-Diethylamine- Benzene (80:15:5)	Silica Gel	0.43	93

Table II

TLC of Biological Tissues

<u>Tissue</u>	<u>Read-Outs</u>			<u>Analysis</u>		<u>Reference</u>	
	<u>Spray</u>	<u>Reagents</u>	<u>UV</u>	<u>M.S.</u>	<u>Quantitative</u>		<u>Qualitative</u>
Urine		X				X	37
Urine		X		X	X		48
Liver		X		X	X		48
Kidney		X		X	X		48
Brain		X		X	X		48
Urine		X				X	31, 32
Whole Blood		X				X	31, 32
Plasma		X				X	31, 32
Serum		X				X	31, 32
Urine		X	X		X		12
Liver		X	X		X		12
Brain		X	X (a)		X		51
Lung		X	X		X		51
Kidney		X	X		X		51
Liver		X	X		X		51
Liver		X	X (b)			X	52
Urine		X	X		X		57

(a) Ultraviolet reflectance photometry

(b) UV and visible spectrophotometry



7.62! Detection Methods

Numerous detection methods, including spray reagents, have been used to visualize trifluoperazine on thin layer plates. A listing of them can be found in Table 12.

Table 12Detection Methods Used in TLC of Trifluoperazine

<u>Reagent</u>	<u>Response (color)</u>	<u>Reference</u>
Phosphomolybdic acid + Ferric chloride	yellow-brown (tan)	86
Folin-Ciocalteu	cameo	32
Ferric chloride in $\text{HClO}_4$ + $\text{HNO}_3$	pink+orange	32,97
Ammonium vanadate	flesh	32
Cinnamylaldehyde	cameo	32
p-Dimethylaminobenzaldehyde	pink	32
Furfural	cameo	32
Iodoplatinate spray	violet	85
Bromine	orange-pink	94
Aniline vapor followed by bromine	mauve-purple	94
Tetracyanoethylene in acetonitrile	brown+yellow	95
2,4,7-Trinitro-9-fluorenone in acetonitrile	grey	95
1,3,5-Trinitrobenzene in toluene	brown	95
Vanadium pentoxide- $\text{H}_2\text{SO}_4$	orange	96
p-Dimethylaminobenzaldehyde	orange	97
40% $\text{H}_2\text{SO}_4$ + heat	orange	92
0.1% Bromocresol purple	blue	98
5% $\text{H}_2\text{SO}_4$ -ethanol	orange	31
$\text{HClO}_4$	brown	99
Nitric acid	brown+yellow	99
5% Ammonium persulfate	orange	52

7.63 High Performance Liquid Chromatography (HPLC)

Adsorption,<sup>100</sup> ion-exchange,<sup>100,103</sup> reverse phase,<sup>101</sup> and ion-pairing reverse phase<sup>102</sup> systems have been used to evaluate trifluoperazine. The procedure is rapid, yielding good resolution of several phenothiazines.

Table 13

HPLC Parameters for Trifluoperazine

<u>Column</u>	<u>Mobile Phase</u>	<u>Flow Rate (ml/min)</u>	<u>Detector</u>	<u>Rt (min) (approx)</u>	<u>Reference</u>
S11-X-1 (Perkin-Elmer)	Chlorobutane:iso-octane containing 1% diethylamine	1	UV (254 nm)	16	100
10N-X-SC	0.01M (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> in methanol: H <sub>2</sub> O (2:3), adjusted to pH 9.0	1	UV (254 nm)	8	100
Ultrasphere-IP (Altex)	0.01M KH <sub>2</sub> PO <sub>4</sub> + 0.01M Nonylamine, adjusted to pH 3.0, + 35% acetonitrile	2.0	UV	5	101
μ Bondapak C-18 (Waters)	10% 0.25M Camphorsulfonic acid, 60% methanol, 30% water, adjusted to pH 3.0	1	UV (262 nm)	30	102
Alkylsulfonic Acid strong cation exchanger	Methanol-0.5M Ammonium Nitrate (pH 6.0) (4:1)	1	UV (254 nm)	10	103

7.64 Gas Liquid Chromatography

Gas liquid chromatography continues to play an important role in the detection and determination of trifluoperazine and its detection and determination of trifluoperazine and its metabolites in biological materials. In Table 14 are listed a number of substrates and other parameters used to chromatograph trifluoperazine.

Using pyrolysis techniques, Fontan, et al<sup>111</sup> could readily identify trifluoperazine and differentiate it from its metabolites and other phenothiazines. De Leenheer<sup>112</sup> coupled preparative gas liquid chromatography with micro-infrared spectroscopy for the identification of trifluoperazine and other phenothiazines. Trifluoperazine was separated on a 1% FFAP column at 230°C.

Table 14Gas Liquid Chromatography

Parameters for the GLC of Trifluoperazine

<u>Column</u>	<u>Column Temperature</u>	<u>Carrier Gas</u>	<u>Detector</u>	<u>Rt (min)</u>	<u>Ref.</u>
5% QF-1 on Anakrom ABS, 100/110	210° for 18 min. programmed to 240°	N <sub>2</sub>	FID	22	104
3.5% XE-60 on Gas Chrom Q, 100/120	235°	He	FID	9.0	104
3% OV-17 on Gas Chrom Q	235°	He	FID	22.5	104
5% OV-1 on Diatoport S, 80-100 mesh	230°	N <sub>2</sub>	FID	6.9	9
2% FFAP on Diatoport S, 80-100 mesh	230°	N <sub>2</sub>	FID	13.6	9
3% SE-30 on Gas Chrom Q, 80-100 mesh	210	He	FID	8.6	85
1% HI-EFF-8BP + 10% SE-52 on Gas Chrom Q, 80-100 mesh	220°	N <sub>2</sub>	FID	~ 120	105
2% SE-30 on Gas Chrom Q, 80-100 mesh	205°	Argon	90SR	42	106
3% OV-1 on Gas Chrom Q, 80-100 mesh	245°	N <sub>2</sub>	FID	3.1	107
5% SE-30 on Diatoport S, 60-80 mesh	270°	N <sub>2</sub>	FID	5.5	108
10% SE-30 and 1% tri-stearin on Gas Chrom W	245°	N <sub>2</sub>	FID	6.9	109
1% HI-EFF-8B on Silanized Gas Chrom P	220°	N <sub>2</sub>	FID	10.9	110
1% HI-EFF-8B on Silanized Gas Chrom P	250°	N <sub>2</sub>	FID	1.6	110

### 7.65 Electrophoresis

Paper electrophoresis on buffered Whatman 3MM paper (pH 3.3 to 9.3) separated trifluoperazine and its sulfoxide.<sup>33</sup> Identification was made from the respective migration distance and by the response to a sulfuric acid spray reagent and by its fluorescence.

pH	Migration (in cm)	
	<u>Trifluoperazine</u>	<u>Sulfoxide</u>
3.3	7.0	7.2
4.7	4.4	6.2
6.0	4.7	5.8
7.2	4.7	5.4
8.0	3.2	5.4
9.3	1.7	5.9

## 8. Miscellaneous

### 8.1 Adsorption Isotherm

Adsorption isotherms of trifluoperazine by carbon black, graphite, silica gel, and polyethylene determined by Nogami, *et al*<sup>114</sup> showed a relationship to its neuroleptic and haemolytic activity. The amount absorbed was related to the molecular volume of R at the 10-position, the bulkiness of the substituent at the 2-position, the pH of the buffer solution, and the partition coefficient in  $\text{CHCl}_3/0.1\text{N HCl}$ . Sorby, *et al*<sup>115</sup> determined the adsorption isotherms by kaolin, talc, and activated carbon. They showed that the adsorption by kaolin and talc was dependent upon the pH of the medium whereas this was not the case with activated carbon.

### 8.2 Surface Activity

Several phenothiazines have been evaluated for their effect on surface activity as an explanation for their physiological activity. Zograf and Munski<sup>116</sup> showed that trifluoperazine was many times more effective than chlorpromazine in lowering the surface tension of a pH 5.0, ionic strength 0.1, solution at 25°C. Seeman and Bialy<sup>117</sup> attributed the activity of tranquilizers to the lowering of the surface tension at the erythrocyte surface via the adsorption of a monomolecular layer of the phenothiazine analog. Trifluoperazine was shown to be significantly more effective in lowering the surface tension than chlorpromazine, and, therefore, a more potent tranquilizer.

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# GRISEOFULVIN

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## GRISEOFULVIN

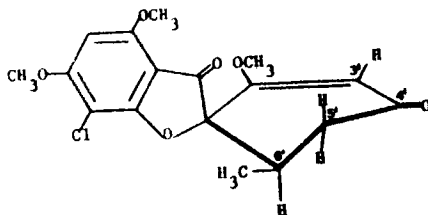
### 1. Description

#### 1.1 Nomenclature

1.1.1 Other names: Curling Factor.

#### 1.2 Formulae

##### 1.2.1 Structural



Various structural formulae have been proposed for griseofulvin, but the currently accepted is that suggested by Grove et al (1). According to this formulae the molecule of the antibiotic contains three rings: the aromatic benzene ring (A), a 5-membered heterocyclic ring with an atom of oxygen (B) and a hydro-aromatic 6-membered ring (C). The A & B rings are condensed forming a coumarone system. Carbon atom 2, which is common rings B & C is an assymetrical carbon atom giving griseofulvin its spiran structure and causing its optical activity, to

which the assymetrical 6 carbon also contri-  
-butes. The C ring may be regarded as the  
methyl ether of the enol form of 2,4 dike-  
tone or as the methyl ether of 6-methyl  
dihydroresorcinol.

### 1.2.2 Wiswesser Line Notation

T56 BOXVJ F01 H01 1G  
C - & DL6V DX  
BUTJ C01 E1

### 1.2.3 Conformation

The preferred conformation of griseofulvin  
in solution is that shown in the stereo-  
structure given before (2). This is based  
on the finding of relatively strong coup-  
ling ( $J = 13.5$  Hz) between the 6 $\alpha$  and the  
5 $\beta$ -protons. This relies on the application  
of an NMR shift reagent [Tris - (Dipevalo-  
methanato) Europium], to the spectrum of a  
partially deuterated sample of griseoful-  
vin.

## 2. Physical Properties:

### 2.1 Crystal Properties:

#### 2.1.1 Crystallinity

Griseofulvin crystallizes from benzene as  
stout octahedra or rhombs. The crystals

are generally up to 5 nm in maximum dimension, although larger particles which may occasionally exceed 30nm may be present. Crystal size affects the absorption of griseofulvin when administered orally.

Microsize griseofulvin may be administered in significantly smaller doses than the conventional size powder to obtain the same effect. The U.S.P. specifies that the official product is the "Microsize" powder (3).

Brown and Sim (4) carried out a quantitative X-ray study of 5-bromogriseofulvin in order to define unambiguously the stereochemical relationship of the 2- and the 6'-centre. Crystals of 5-bromogriseofulvin belong to the monoclinic system, space group  $P2_1(C_2^2)$ , with two molecules of  $C_{17}H_{16}BrClO_6$  in a unit cell of dimensions  $a = 10.96$ ,  $b = 8.61$ ,  $c = 10.27 \text{ \AA}$ ,  $\beta = 108^\circ 30'$ . Initial phase determination was based on the bromine and the chlorine atom and several three dimensional Fourier syntheses were evaluated, followed by least squares refinement of the atomic parameters. The

final discrepancy R over the 1129 observed reflexions is 14%.

## 2.2 Dissolution

The dissolution rate of griseofulvin had been significantly enhanced by solid dispersion in succinic acid. This had been initially attributed to the extensive formation of a solid solution of griseofulvin in succinic acid (5). Later, it was shown by X-ray diffraction and differential thermal analysis methods that solid solubility was negligible and such a binary system could be classified more adequately as a simple eutectic mixture (6). The dissolution profile of the griseofulvin-succinic acid eutectic mixture system was evaluated using the powder and constant surface area tablet methods (7). Contrary to the original proposal of Sekigushi *et al* (8), dissolution rates of griseofulvin from solid dispersions were found to be markedly affected by their particle size.

## 2.3 Spectral Properties

### 2.3.1 Ultraviolet Spectrum

In ethanolic solution, griseofulvin exhibits a characteristic UV spectrum (Fig.1)

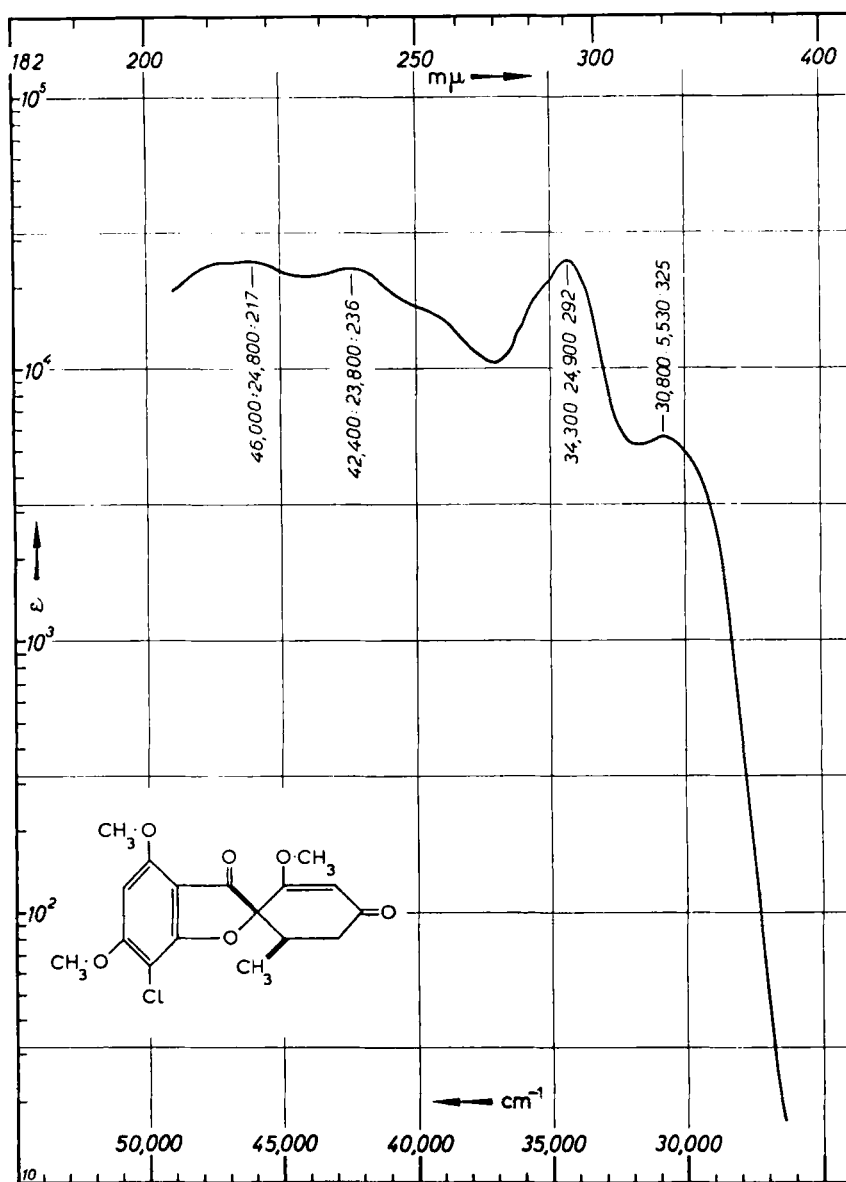


Fig. 1 - UV Spectrum of Griseofulvin in Ethanol.

with maxima at 325, 292 and 236 nm. The spectrum of isogriseofulvin is similar to that of griseofulvin differing only by the presence of a fourth maximum at 263 nm.  $E_{1\text{cm}}^{1\%}$  at 292 nm = 686. The UV spectral data of griseofulvin analoges have also been reported (9-11).

### 2.3.2 Nuclear Magnetic Resonance Spectra

#### 2.3.2.1 PMR

The proton magnetic resonance spectra of griseofulvin and its derivatives have been investigated(12, 14).

A typical PMR spectrum of griseofulvin is shown in Fig.2. The sample was dissolved in deuterated chloroform ( $\text{CDCl}_3$ ). The spectrum was recorded on a Varian T-60A NMR spectrometer with TMS as the reference standard. The following structural assignments have been made (15).

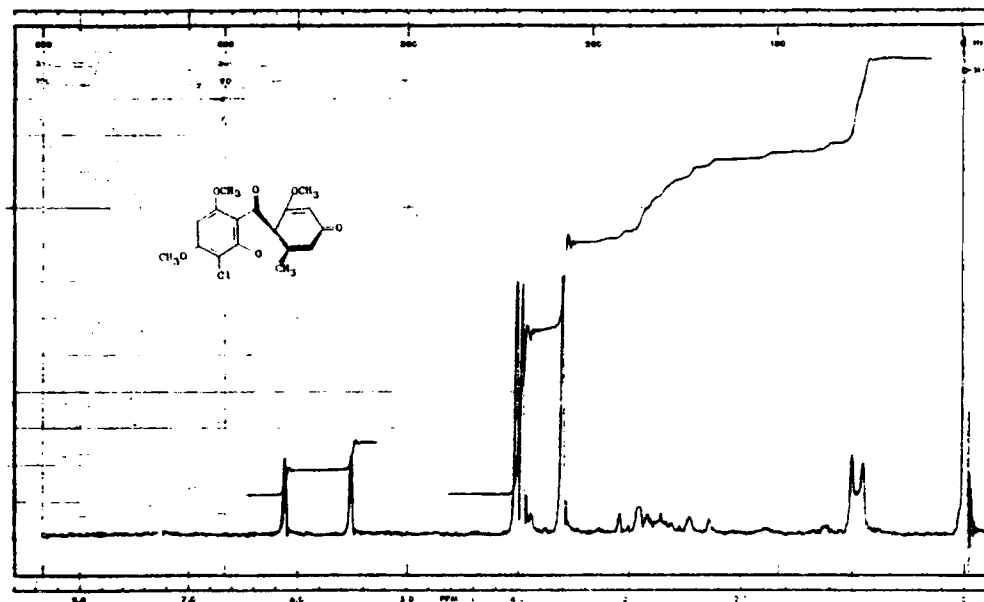


Fig. 2 - PMR Spectrum of Griseofulvin in  $\text{CDCl}_3$  and TMS.

<u>Chemical Shift(<math>\delta</math>)</u>	<u>Assignment</u>
0.97 (doublet)	6' - CH <sub>3</sub>
2.70 (multiplet)	5' -, 6' - H
3.60 (singlet)	2' - OCH <sub>3</sub>
3.97 (singlet)	4 - OCH <sub>3</sub>
4.00 (singlet)	6 - OCH <sub>3</sub>
5.50 (singlet)	3' - H
6.13 (singlet)	5 - H

The PMR spectrum of griseofulvin -5', 5'-d<sub>2</sub> exhibits only one aliphatic proton appearing as a quartet at 2.75  $\delta$  ascribable to the 6'- $\alpha$ -proton (2). On stirring with neutral alumina in chloroform this compound undergoes stereoselective partial replacement of the 5'- $\beta$ -deuterium substituent with hydrogen to give griseofulvin 5'- $\alpha$ -d. The PMR spectrum of a mixture of the 2 compounds (Fig.3-A) shows no peaks in the region of 2.3 (5 $\alpha$ -H) but exhibits a complex band at 2.7 - 2.9 $\delta$  (1,4H) representing the coupled and closely spaced 5'- $\beta$ - and



$6'/\alpha$  proton signals. A strikingly altered PMR spectrum (Fig. 3) was obtained on application of  $\text{Eu}(\text{DPM})_3$ . Proton signals are shifted downfield in general proportion to their closeness to the C-4 carbonyl oxygen. The signals due to  $6'/\text{-CH}_3$  (1.48),  $6'/\alpha\text{-H}$  ( $\sim 3.98$ ), and  $5'/\beta\text{-H}$  (5.3 $\delta$ ) constitute a first order ( $A_3\text{MX}$ ) system in which the doublet at 5.3 $\delta$  gives  $J\ 5'/\beta\text{-}6'/\alpha \sim 13.5$  Hz. A vicinal coupling of this magnitude must be due to trans diaxial hydrogen substituent.

The PMR spectrum of griseofulvin in  $\text{DMSO } D_6$  has been reported (16).

### 3. Synthesis

Several synthetic routes to griseofulvin have been reported (17-20).

Fig. 3-A

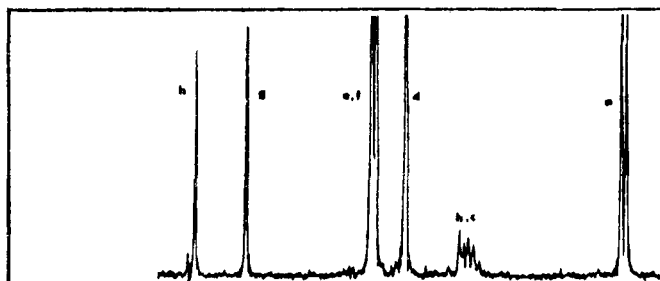
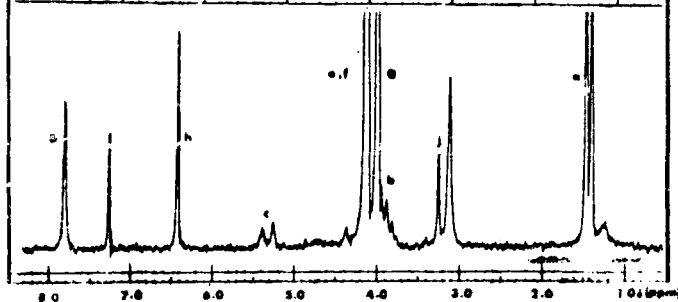


Fig. 3-B



## PROTON ASSIGNMENTS

- |                           |  |
|---------------------------|--|
| a. 6'-CH <sub>3</sub>     | g. 3'-H  |
| b. 6'-H                   | h. Ar-H  |
| c. 3'-H                   | i. CHCl <sub>3</sub>                               |
| d. 2'-OCH <sub>3</sub>    | j. bands due to Eu(DPM) <sub>3</sub> <sup>18</sup> |
| e, f. Ar OCH <sub>3</sub> |  |

Fig. 3-A : NMR (100) Spectrum of Griseofulvin-5', 5'/d<sub>2</sub> and its stereoselective Hydrogen Exchange Product in CDCl<sub>3</sub>.

Fig. 3-B : The same with 0.4 molar equivalent of Eu (DPM)<sub>3</sub> in CDCl<sub>3</sub>.

#### 4. Methods of Analysis

##### 4.1 Time-resolved Phosphorimetry

Phosphorescence life times of griseofulvin and dechlorogriseofulvin are shown to be 0.11 sec. and 1.16 sec. respectively (22). This 10-fold difference was shown to enable the use of time-resolved phosphorimetry for the determination of griseofulvin in mixtures with dechlorogriseofulvin.

##### 4.2 Liquid Chromatography

###### 4.2.1 Column Chromatography

A liquid solid chromatographic method was reported (23) for the direct analysis of griseofulvin in complex fermenter brothes. The method is tedious and time consuming.

##### 4.3 Isotope Dilution

Ashton (24) described an isotope dilution method for the assay of griseofulvin based on the estimation of the radioactivity employing griseofulvin labelled with radioactive  $^{36}\text{Cl}$ . McNall (25,26) reported, another method using tritium-labelled griseofulvin.

#### 5.4 PMR Spectrometry

A rapid, accurate and specific PMR method for the determination of griseofulvin in bulk drug and pharmaceutical formulations has been developed in our laboratory (15). From Fig.2, it is evident that griseofulvin exhibits, among other peaks, two singlets at 3.97 and 4.00 ppm (in  $\text{CDCl}_3$ ) assigned to the 4'- and 6'- methoxy protons respectively. Since the integration of these two peaks gives the largest region for measurement, they are chosen for the quantitative analysis of griseofulvin.

Acetanilide, exhibiting a three protons singlet at 2.30 ppm (in  $\text{CDCl}_3$ ), assigned to its methyl groups is employed as internal standard. The determination is based on the integration of the 4'- and 6'-methoxyprotons of griseofulvin relative to that of the methylprotons of acetanilide. Accurate determination is achieved, since the signals chosen for griseofulvin are widely separated from that of acetanilide. Ethanol-free chloroform is used as the solvent, its proton singlet at 7.25 ppm does not interfere with the upfield protons of both compounds. Fig. 4.

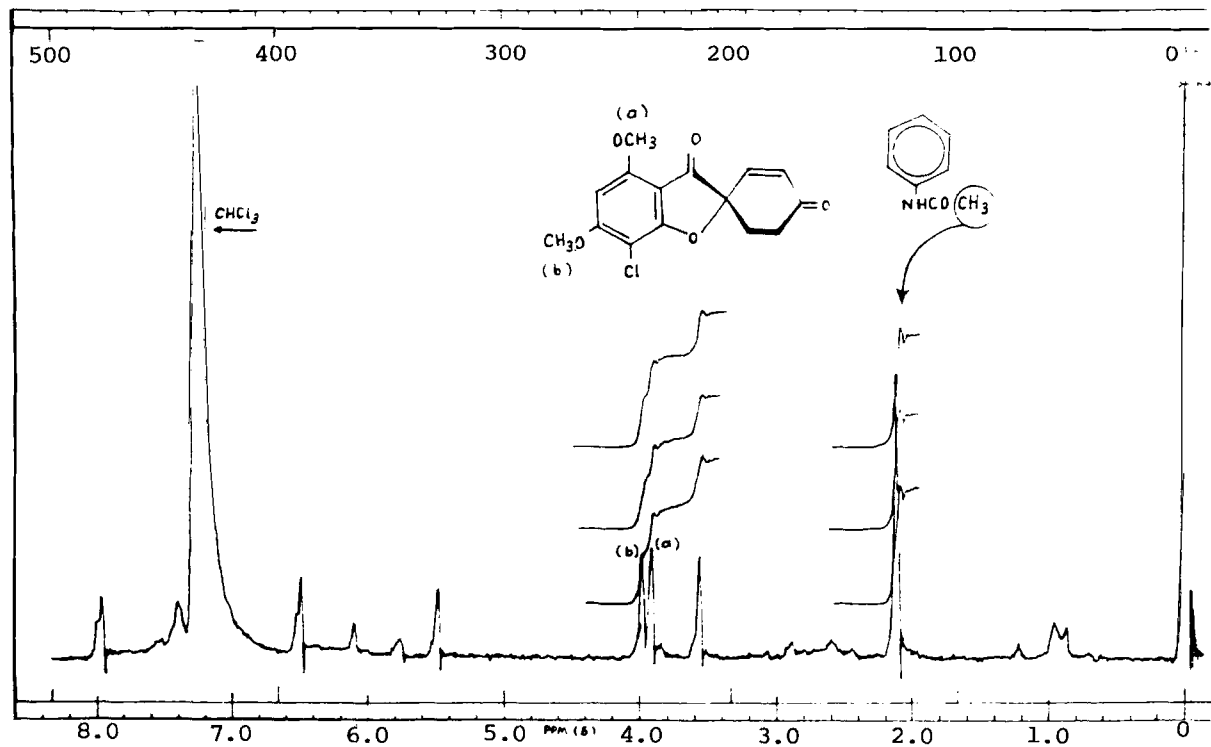


Fig. 4 - PMR spectrum of Griseofulvin, acetanilide and TMS in ethanol-free chloroform.

Assay of a series of known standard mixtures of griseofulvin and acetanilide by this PMR technique established the accuracy and precision of the method with an average recovery of 99.55%. The results of estimation of griseofulvin in tablets and drysuspension powders are in agreement with pharmacoepial requirements. No interference from excipients could be observed.

#### 4.5 Microbiological

Dittmer (27) reported on the determination of microbiological activity of griseofulvin in body fluids by dilution methods in liquid or solid media using *Tricophyton mentagrophyte* as the test organism.

Mrtek et al (28) developed the microculture slide technique of Elliott et al (29). The assay system consists of a suspension of *Microsporum gypseum* macroconidia in Sabouraud liquid medium containing nanogram quantities of griseofulvin. Antifungal activity is determined on specially prepared microculture slides by measuring changes in the rate of hyphal elongation. A liner relationship of log dose to hyphal growth rate is

observed in the range of 0.001 - 0.01 mcg/ml griseofulvin. This technique exhibited precision at least equivalent to that of the agar cup procedure.

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# METHADONE HYDROCHLORIDE

*Mahmoud A. Hassan and Abdullah A. Al-Badr*

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METHADONE HYDROCHLORIDE1. Description1.1 Nomenclature1.11 Chemical Name

N,N-Dimethyl-1, 1-diphenyl-1-propan-1-one-methyl  
propylamine hydrochloride (1).

1.12 Generic Name

Methadone hydrochloride; Metadone hydrochloride.

1.13 Trade Name

Tussal.

1.14 Wiswesser Line Notation

2VXR&R&1Y&N1&1 &GH DL

1.2 Conformation

A probable conformation of methadone hydrochloride, based upon crystallographic (2) and spectroscopic evidence (3), is shown in Fig.1. This conformation is stabilised by a hydrogen-bonding interaction as has been suggested by Beckett and Casy (4). Further evidence of such conformation was also obtained by the work of Henkel et al (5).

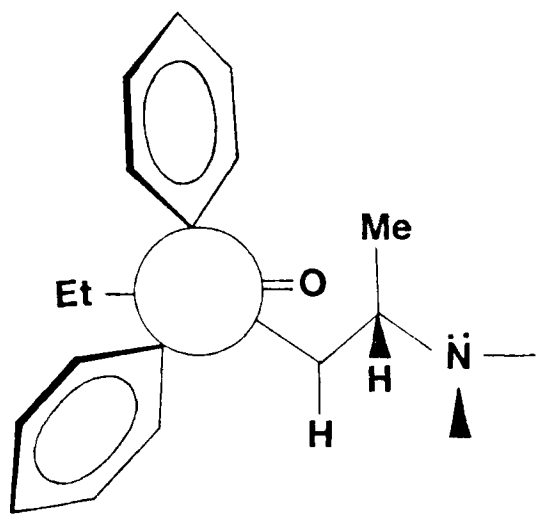
2. Physical Properties2.1 Optical Rotatory Dispersion Spectrum

The ORD characteristics of (+) and (-)-methadone have been reported (1) and given below. The ORD curves are shown in Fig. 2.

(+) Methadone :

$[\alpha]_D^{25} +34^\circ$  (Cyclohexane). RD(C, 0.15; Dioxane):  
 $[\Phi]_{600} + 164^\circ$ ;  
 $[\Phi]_{500} +164^\circ$ ;  $[\Phi]_{400} + 83^\circ$ ;  $[\Phi]_{375} - 0^\circ$ ;  $[\Phi]_{314} -4079^\circ$ ;  
 $[\Phi]_{300} \pm 0^\circ$ ;  $[\Phi]_{274} + 17\ 394^\circ$ ;  $[\Phi]_{270} +15\ 271$ ;  
 $[\Phi]_{267} + 15\ 904^\circ$ ;  $[\Phi]_{254} +12\ 725^\circ$ ;  $[\Phi]_{230} + 13\ 995^\circ$ .

UV :  $\lambda_{\max} = 286\text{ nm}$  ( $\log \epsilon = 2,62$ );  $296\text{ nm}$  ( $\log \epsilon = 2,61$ ).



PROBABLE CONFORMATION OF  
METHADONE

FIG. 1

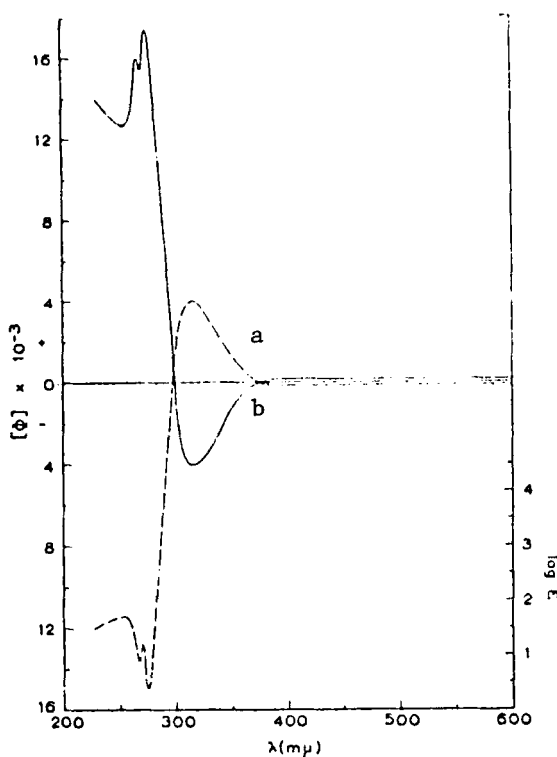


Fig. 2: ORD Curves of (+)-methadone (a) and (-)-methadone (b),

(-)- Methadone :

$[\alpha]_D -36^\circ$  (Cyclohexane). RD (C, 0, 11; Dioxanne):  $[\Phi]_{600} -185^\circ$ ;  $[\Phi]_{500} -185^\circ$ ;  $[\Phi]_{400} -93^\circ$ ;  $[\Phi]_{375} \pm 0^\circ$ ;  $[\Phi]_{316} + 4.066^\circ$ ;  $[\Phi]_{298} \pm 0^\circ$ ;  $[\Phi]_{274} - 14.974^\circ$ ;  $[\Phi]_{270} -12.796^\circ$ ;  $[\Phi]_{268} -13.611^\circ$ ;  $[\Phi]_{254} -11.433^\circ$ ;  $[\Phi]_{228} - 11.980^\circ$ .

UV :  $\lambda_{\text{max}} = 250 \text{ nm}$  ( $\log \epsilon = 3.01$ ) (inflexion),  
 286 nm ( $\log \epsilon = 2.62$ ), 296 nm ( $\log \epsilon = 2.62$ ).

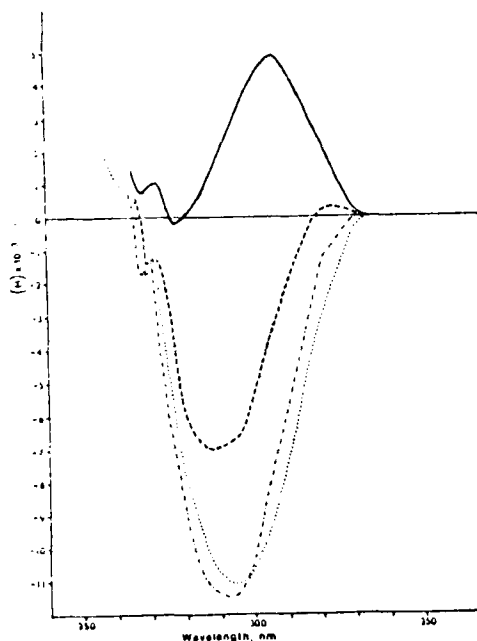


Fig. 3: CD spectra of (+)-(6S)-methadone: 0.1% solutions in  $\text{CH}_3\text{OH}$  (—),  $\text{CHCl}_3$  (---), hexane(···), and  $\text{CH}_3\text{CN}$ (-·-·).

## 2.2 Circular Dichroism Spectrum

The CD spectral characteristics for (+)-methadone and (+)-methadone hydrochloride have been reported (5) and are given below. The CD spectra are shown in Fig. 3, and Fig. 4.

(+)-(6S)-Methadone: CD (c 0.1, hexane)  $[\theta]_{330-310}$ ,  $[\theta]_{320-2970}$ ,  $[\theta]_{310-7180}$ ,  $[\theta]_{300-10,520}$ ,  $[\theta]_{294-11,080}$ ,  $[\theta]_{290-10,077}$ ,  $[\theta]_{280-7920}$ ,  $[\theta]_{271-1350}$ ,  $[\theta]_{269-1390}$ ,  $[\theta]_{2670}$ ,  $[\theta]_{264-1040}$ ,  $[\theta]_{262-940}$ ; CD (c 0.1,  $\text{CHCl}_3$ )  $[\theta]_{330-120}$ ,  $[\theta]_{323-300}$ ,  $[\theta]_{320-190}$ ,  $[\theta]_{318-0}$ ,  $[\theta]_{310-1980}$ ,  $[\theta]_{300-5320}$ ,  $[\theta]_{289-6980}$ ,  $[\theta]_{280-5820}$ ,  $[\theta]_{272-1420}$ ,  $[\theta]_{267-0}$ ; CD (c 0.1,  $\text{CH}_3\text{OH}$ )  $[\theta]_{330-230}$ ,  $[\theta]_{320-2320}$ ,  $[\theta]_{310-4490}$ ,  $[\theta]_{307-4870}$ ,  $[\theta]_{300-4100}$ ,  $[\theta]_{290-1860}$ ,  $[\theta]_{280-0}$ ,  $[\theta]_{277-230}$ ,  $[\theta]_{272+1080}$ ,  $[\theta]_{268-740}$ ,  $[\theta]_{265-1480}$ ,  $[\theta]_{260-1110}$ ; CD (c 0.1,  $\text{CH}_3\text{CN}$ )  $[\theta]_{330-0}$ ,  $[\theta]_{320-1240}$ ,  $[\theta]_{310-5570}$ ,  $[\theta]_{300-10,090}$ ,  $[\theta]_{292-11,460}$ ,  $[\theta]_{290-11,370}$ ,  $[\theta]_{280-8940}$ ,  $[\theta]_{271-1485}$ ,  $[\theta]_{269-1700}$ ,  $[\theta]_{267-0}$ .

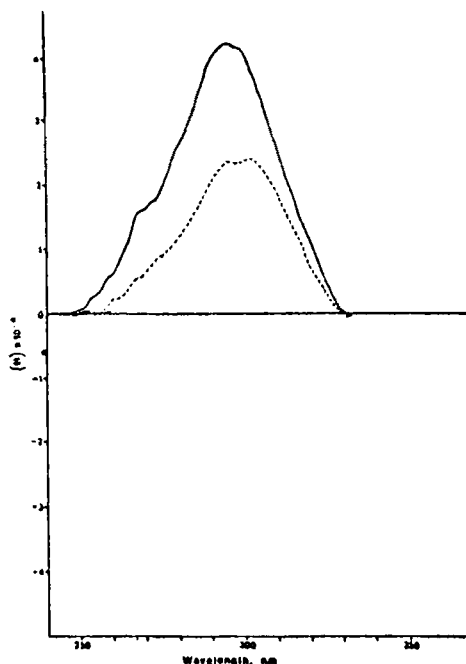


Fig. 4: CD spectra of (+)-(6S)-methadone hydrochloride, 0.025% solutions in  $\text{CH}_3\text{OH}$  (—) and  $\text{CHCl}_3$  (---).

(+)-(6S)-Methadone Hydrochloride: CD (c 0.025,  $\text{CHCl}_3$ )  
 $[\theta]_{330}$  0,  $[\theta]_{320}$  5900,  $[\theta]_{310}$  17,300,  $[\theta]_{302}$  24,000,  
 $[\theta]_{298}$  23,400,  $[\theta]_{295}$  23,700,  $[\theta]_{290}$  20,400,  $[\theta]_{280}$  11,700,  
 $[\theta]_{275}$  9100,  $[\theta]_{274}$  8900,  $[\theta]_{270}$  6600,  $[\theta]_{268}$  5700,  
 $[\theta]_{267}$  5500,  $[\theta]_{262}$  2500,  $[\theta]_{260}$  2300,  $[\theta]_{255}$  600,  
 $[\theta]_{254}$  600,  $[\theta]_{252}$  0; CD (c 0.1  $\text{CH}_3\text{OH}$ )  $[\theta]_{330}$  200,  
 $[\theta]_{320}$  6800,  $[\theta]_{310}$  25,800,  $[\theta]_{300}$  40,800,  $[\theta]_{295}$  42,100,  
 $[\theta]_{290}$  39,400,  $[\theta]_{280}$  26,200,  $[\theta]_{273}$  17,800,  $[\theta]_{272}$  17,600,  
 $[\theta]_{270}$  16,300,  $[\theta]_{267}$  11,700,  $[\theta]_{265}$  11,500,  $[\theta]_{260}$  6100,  
 $[\theta]_{258}$  5500,  $[\theta]_{255}$  3000,  $[\theta]_{253}$  2400,  $[\theta]_{247}$  0.

### 2.3 Crystallographic Properties

Hanson and Ahmed (2) have reported the crystal structure and absolute configuration of monoclinic form of d-methadone hydrobromide. The crystal is monoclinic, probably  $P_{21}$ ,  $a = 10.69$ ,  $b = 8.74$ ,  $c = 10.74$

$A^\circ, B = 94.6^\circ, Z = 2$ . The structure determination, which was essentially three-dimensional, was begun by the heavy atom method, and completed by means of differential syntheses. The absolute configuration of the molecule was determined by measuring the effect on two selected sets of reflexions of the imaginary part of the dispersion of copper radiation by the bromine atom. A projection of a single molecule along a convenient direction is shown in Fig.5. The absolute configuration is that of the (+)-isomer. The bromine atom, which is not shown, lies near the apex of the pyramid formed by the nitrogen atom and its neighbours.

### 3. Methods of Analysis:

#### 3.1 Gravimetric Analysis:

Loucas et al (6) have published a gravimetric method for the determination of methadone hydrochloride in flavoured syrup formulation, by mixing the sample (equivalent to 10-20 mg of the drug) with 10ml of 1% Molybdophosphoric acid solution, collecting the precipitate on a Millipore membrane-filter and drying it at  $60^\circ$ ; 1mg of the precipitate = 0.4mg of the drug. Nitrogenous bases, particularly alkaloids interfered, being co-precipitated with the drug.

#### 3.2 Ultraviolet Analysis:

Caddy et al (7) described an oxidative analytical procedure for the determination of certain drugs containing the diphenylmethylene group in blood and urine. The method is based on the oxidation of the drug with alkaline potassium permanganate to form benzophenone. For calibration, a standard solution of the drug salt is heated with alkaline potassium permanganate solution and heptane, and the extinction of the organic layer is measured vs heptane at (247 nm). Beers Law is obeyed for up to 20  $\mu$ g of benzophenone per ml of heptane solution. Urine or blood samples (adjusted to pH 10.5) are extracted with ethyl ether the extract is washed with N HCl, and the concentrated acid solution is treated as for standard solution.

#### 3.3 Ion-Exchange Chromatography:

Knox et al (8) have described a chromatographic method for the separation of methadone from mixtures of other



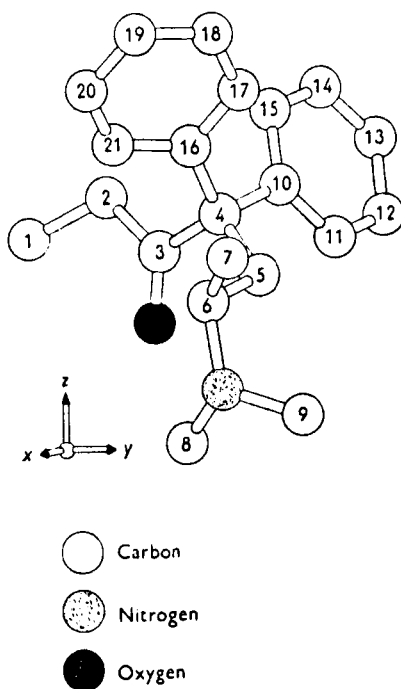


Fig. 5: The d-methadone molecule, as it occurs in the monoclinic form of the bromine derivative. The orientation triplet is composed of 1  $\text{\AA}^\circ$  vectors, in the directions of the principal axes.

drugs. The method was carried out on a stainless steel column (1m x 2.1mm) which was packed with Zipax SCX (37-44  $\mu$ m), and sample was injected through a septum and was eluted with aqueous borate buffer under pressures of 500-1500 lb per square inch; the elute was passed through an 8  $\mu$ l flow-cell and its extinction was measured. Methadone was separated with buffer solution of pH 9.8.

#### 3.4 Radio-Immunoassay:

Cleeland et al (9) published a review dealing with the analysis of urine, blood, saliva and tissues for methadone with other drugs of abuse using radio-immunoassay.

#### 3.5 Thin Layer Chromatography (TLC):

Gupta et al (10) have described a TLC method for screening of the major methadone metabolites and methyl amphetamine in urine. Urine 1ml is placed in a screw-capped PTFE-lined culture tube and 0.25 M  $\text{CuSO}_4$  (1ml), saturated aqueous sodium bicarbonate (1ml) and chloroform (5ml) and added. The aqueous layer after centrifugation is aspirated off and the organic layer is decanted into a test-tube to which is added 4-chloro-7-nitrobenzofurazan chloride in chloroform. The solution is evaporated to dryness and the residue is dissolved in chloroform. The solution is subjected to TLC on silica gel (0.25 mm thick) by development with ethylether-benzene(1:1). The methadone metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine produces a blue-green and purple spots ( $R_F$  0.94 and 0.84, respectively) with the above reagent.

Jain et al (11) have reported another TLC method for the separation of methadone and its primary metabolite in the presence of other drugs in urine specimens. The sample was treated with conc. aqueous ammonia and extracted with chloroform-ethyl acetate-methanol (3:1:1). The organic layer was filtered through phase-separating paper and evaporated at 70° under  $N_2$ . The residue was dissolved in methanol and applied to a silica gel E or FG precoated plates. The best solvent systems were ethyl acetate-dichloromethane-conc. aqueous ammonia (90:10:0.9), ethyl acetate-octanol-conc. aqueous ammonia (93:7:1) and ethylacetate-isopropylether-water-conc. aqueous ammonia (90:10:1.1). Spots were detected with iodoplatinate spray reagent. Methadone and its primary metabolite 2-ethylidene-1, 5-dimethyl-3,

3-diphenyl pyrrolidine were well separated from each other. The limit of detection was 0.25 µg/ml both for methadone and for its metabolite.

Davis *et al* (12) have reported an improved thin layer chromatographic system for methadone and its metabolites in biological samples using the Gelman instant thin layer chromatography (ITLC) system. The ITLC was modified by applying a thicker layer of silica gel to the base of the imprignated fiber-glass strip, so as to reduce the tendency to over load when working with biological extracts. The technique described is illustrated by the application to the separation of labelled methadone and metabolites (pyrrolidine and the N-oxide) in a kidney extract by the following solvent systems:

a) ethylacetate-methanol-aqueous ammonia

17 : 2 : 1

b) benzene-ethylacetate

19 : 1

c) benzene-ethylacetate-methanol-aqueous ammonia

800 : 2000 : 12 : 1

followed by radiometric coating

### 3.6 Gas Chromatography:

Gas liquid chromatography systems for determination of methadone in sustained-release tablets (13). The method involves the extraction of a tablet at 37° with successive portions of dissolution medium (mixtures of gastric fluids and intestinal fluids of pH increasing from 1.2-7.5). Each extract is made alkaline to phenolphthalein and 10 ml portions were extracted with chloroform (50 ml). Each chloroform extract was dried over sodium sulfate and a 10 ml portion was evaporated with a chloroform solution of atropine (internal standard). The residue was dissolved in chloroform (2 ml) and a 1-2 µl portion was subjected to GLC on a spiral siliconized glass column (3 ft. long x 2 mm packed 3% of SP 2250-DP on Supelcophrt (100-120 mesh) and operated at 235° with a Helium 35 ml min<sup>-1</sup> as a carrier gas and flame ionization detection. The amount of methadone was calculated

from the peak height and molar response ratios relative to atropine.

Lynn *et al* (14) has reported a new gas-chromatographic assay for determination of methadone in man and animals (6). The internal standard, 2-dimethylamino-4-4-diphenylnonane-5-one is added to the specimen containing the drug and then extracted with chlorobutane at pH 9.8. Then it is extracted into 0.5M H<sub>2</sub>SO<sub>4</sub> and after alkalization is extracted into chloroform. The extract was analysed on a column (6ft x 2mm) of 1.5% OV-101 on Gas-Chrom Q (100-120 mesh). The temperature is programmed from 170-250° at 1 ° min<sup>-1</sup>, with N<sub>2</sub> as carrier gas (30ml min<sup>-1</sup>) and a H-flame ionization detector. The peak area ratio of the standard and the drug was obtained by electronic integration. Tracer studies with (±)-<sup>14</sup>C-methadone showed that the recovery was 93 ± 2% for the extraction and > 99% in subsequent stages.

### 3.7 High Pressure Liquid Chromatography (HPLC):

Knox and Jurand (15) have applied a high-speed liquid chromatography for the determination of methadone and other narcotics. The chromatographic behaviour of the narcotics studied has been investigated on a glass or stainless steel column (80-100 cm x 2mm) packed with Zipax Pellicular resins (37-44 m) and operated at room temperature, with UV detection. Conditions are outlined for rapid determination of methadone on a column of strong anion exchange resin. The eluted compound was identified by its UV absorption and mass spectrum.

Trinler and Renland (16) have reported a rapid screening of methadone and other narcotics by reverse phase HPLC. The column (2ft x 0.125 inch, o.d.) packed with Bondapak C<sub>18</sub> - Corasil; detection is by UV spectrometry (254 nm). The eluent is acetonitrile-water (9:1) and the fractions are collected for analysis by UV or IR spectrometry.

Goodman *et al* (17) have tried a combination of HPLC and tritium exchange for the determination of common drugs of abuse and their metabolites including methadone. The HPLC effluent is passed through the tritium exchange system, which consisted of a PTFE-lined stainless-steel column packed with a tritium exchange

polymer followed by an ionization chamber detector. The method was partially successful.

Hsieh *et al* (18) have recently reported a high-performance liquid chromatographic analysis of methadone in sustained release formulations. HPLC separation of methadone was carried out using a reversed-phase  $\mu$  Bondapak C<sub>18</sub> column. The column temperature was ambient. The electrometer was set at 0.01 a.u.f.s. with a recorded chart speed of 2 in. per 10 min. The volume of the samples introduced into the column was 10  $\mu$ l. The solvent (mobile phase) flow rate was controlled at 1.0 ml/min. A stock solution of 0.1 mg/ml anthracene in methanol was used as an internal standard. The sodium salt of 1-pentanesulfonic acid was used as an ion-pair agent. Fig. 6a represents a typical chromatogram of methadone hydrochloride using a mobile phase of methanol-water (75:25), while Fig. 6b illustrates the response of the same solution when the ion-pair agent is present in the mobile phase. It is seen that the ion-pair agent increases the absorption and the resolution of the methadone peak. The high sensitivity and the low quantities ( $\mu$ g) of drug detected by this method indicates that this method may be successfully used for the *in vivo* determination of methadone (Table 1).

Recovery data of methadone from sustained release tablets.

Weight of sample (mg)	Methadone in sample (mg)	Methadone recovered (mg)	Recovery $\pm 5\%$
5	1.1	1.020	93
10	2.2	2.050	93
15	3.3	3.100	94
20	4.4	4.090	93

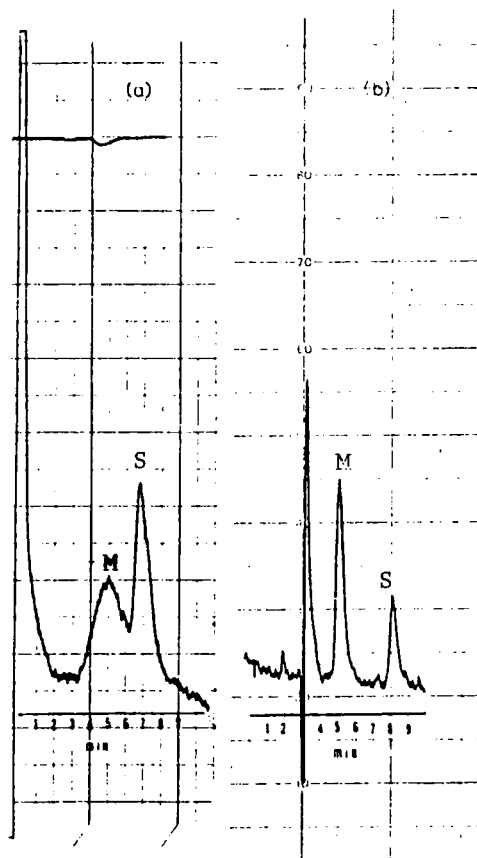


Fig. 6: (a) Typical chromatogram of methadone hydrochloride in a methanol-water (75:25) solution. (b) Chromatogram of methadone hydrochloride in the presence of an ion-pair agent (sodium salt of 1-pentanesulfonic acid). M = Methadone; S = internal standard.

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